Departamento de Biología
Universidad Autónoma de Madrid

Artichoke, a novel LRR protein, localizes to the supporting extracellular matrix of ciliated sensory organs in *Drosophila* and is necessary for its assembly and physiology.

Memoria de Tesis doctoral presentada ante la Facultad de Ciencias,
de la Universidad Autónoma de Madrid, por

Marta Andrés de Miguel

para la obtención del título de Doctora
en el programa de Doctorado Oficial en Biología Celular y Genética.

Trabajo dirigido por

Dr. Inmaculada Canal Beltrán

Dr. Laura Torroja Fungairiño

Madrid, Octubre de 2012

CONTENTS

LIST OF FIGURES

ABBREVIATIONS

atk: *artichoke* gene

ATK: Artichoke protein

ato: *atonal* gene

BCIP: 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt

BF: Best Frequency

CA cell: Cap Attachment cell

cDNA: complementary DNA

ch organs: chordotonal organs

CNS: Central Nervous System

DNA: Deoxyribonucleic Acid

DO: Dorsal Organ

EGFR: Epidermal Growth Factor

es organs: external sensory organs

GFP: Green Fluorescent Protein

GST: Gluthatione-S-transferase

JO: Johnston´s Organ

kni: *knirps* gene

LA cell: Ligament Attachment cell

lch5 organ: lateral pentaloscolopidial organ

LRR: Leucine Rich Repeats

M: Molar

NBT: Nitro-blue Tetrazolium Chloride

nompA: *no mechanoreceptor potential A* gene

nompC: *no mechanoreceptor potential C* gene

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

PNS: Peripheral Nervous System

Rfx: *Regulator Factor X* gene

RI: Response Index

RNA: Ribonucleic Acid

RT-PCR: Reverse Transcription Polymerase Chain Reaction

ru: *roughoid* gene

SOP: Sensory Organ Precursor

TO: Terminal Organ

UAS: Upstream Activated Sequence

Uhg: U22 host gene

VO: Ventral Organ

ZP: Zona Pellucida

WPP: white pre-pupal

Summary

SUMMARY

Cilia are key components of the sensory transduction machinery in many different organisms along the phylogenetic tree, ranging from single-celled eukaryotes to humans. These sensory cilia, also known as primary cilia, appear as projections of the neuronal membrane and are immobile because they lack the pair of central microtubules that confers motility to motile cilia. They function as the structures where the transduction process starts as the ionic channels or receptors located on the distal region of their membranes translate the stimulus energy into receptor potentials that can be understood by the nervous system. Different modalities of channels and receptors confer specificity to each type of sensory receptor, but the cilium, as the structure that bears these channels playing an active role in the transduction process, has been maintained along evolution and appears in many different sensory systems both in vertebrates and invertebrates. In *Drosophila melanogaster* only neurons within mechano- and chemosensory organs possess primary cilia.

Most ciliated sensory neurons are enclosed within sensory organs formed by accessory cells. These cells create an appropriate ionic environment for the transduction to occur and make up the sensory structures that detect and transmit the stimulus to the cilium. Previous studies have shown that accessory cells can influence as well the cilium development, and our study provides new data confirming this idea. Therefore, transduction can be

understood as a close interplay between the sensory cilium and the supporting accessory structures.

In *Drosophila melanogaster* mechano- and chemosensory organs are formed following a series of homologous asymmetric divisions that lead to the formation of the sensory neuron and the accessory cells. The tip of the sensory neuron connects to the distal accessory cells through a supporting extracellular matrix (ECM). In mechanosensory organs this matrix, known as dendritic cap, has been directly involved in mechanotransduction by directly binding the ionic channels and transmitting to them the mechanical energy from the cuticular structures. Morphological defects that affect the dendritic cap impinge mechanotransduction, most likely because of an interruption in the transmission of the stimulus.

Up to date, little is known about the molecular composition of the dendritic cap. Previous studies have shown that NompA, a transmembrane protein with an extracellular *zona pellucida* (ZP) domain, typical of fibrils forming components in different extracellular matrix, is part of the scaffolding that constitutes it (Chung et al. 2001). It is still unknown which are the molecules that interact with NompA in the neuronal membrane or how the dendritic cap connects to the cuticular structures that detect the sensory stimulus.

In our work we describe how Artichoke, a novel protein that bears Leucine-Rich Repeats (LRR) domains, typical of adhesion molecules, is expressed in

all embryonic sensory organs from *Drosophila*, both in mechano- and chemosensory, and localizes to the dendritic cap. ATK is secreted by an accessory cell in each sensory organ, although the nature of this cell varies in the different organ types, implying a specific regulation of its expression. Our morphological analysis of *atk* mutant embryos shows that the ciliated mechanosensory neurons are not properly stretched. To address whether this anatomical defects impinge on the functionality of the sensory neurons we performed both larval locomotor and gustatory tests. Our results show that both sensory modalities are affected in *atk* mutant larvae, entailing that an appropriate coupling of the sensory cilium to the cuticular structures is necessary for the sensory organs to be fully functional, independent of the nature of the stimulus. We demonstrate that accessory cells play an essential role in the organization and functionality of sensory organs and that a supporting ECM is necessary for both mechano- and chemosensation in *D. melanogaster*.

Resumen

RESUMEN

Los cilios son componentes fundamentales de la maquinaria de transducción sensorial en organismos tan diversos como eucariotas unicelulares o humanos. Estos cilios sensoriales o primarios, que aparecen como proyecciones de la membrana neuronal, son inmóviles y carecen del par de microtúbulos central que caracteriza a los cilios móviles. Funcionan como estructuras donde comienza la transducción sensorial gracias a los canales iónicos que se encuentran situados en sus membranas y que transducen la energía del estímulo en potenciales de receptor que son el lenguaje que habla el sistema nervioso. Las diferentes modalidades de canales iónicos o receptores confieren la especificidad para cada tipo de receptor sensorial, pero el cilio en sí mismo, como estructura de soporte para estos canales que juega un papel activo en la transducción sensorial, se ha mantenido a lo largo de la evolución, apareciendo en sistemas sensoriales muy diversos tanto en vertebrados como invertebrados. En *Drosophila melanogaster* sólo los órganos mecano- y quimiosensoriales poseen cilios primarios.

La mayoría de las neuronas ciliadas se encuentran rodeadas por células accesorias. El conjunto formado por la neurona ciliada y las células accesorias que la acompañan constituye un órgano sensorial. Las células accesorias crean el ambiente iónico adecuado para la transducción y construyen las estructuras que detectan el estímulo y lo transmiten hasta

el cilio. Estudios previos han mostrado además que las células accesorias pueden influenciar el desarrollo del cilio y nuestros datos confirman estos resultados. Parece, por tanto, que la transducción sensorial debe ser entendida como un proceso en el que el cilio sensorial y las estructuras accesorias de soporte interaccionan de manera estrecha.

En *Drosophila melanogaster* los órganos mecano- y quimiosensoriales son formados siguiendo una serie de divisiones asimétricas homólogas que dan lugar a la formación de la neurona sensorial y de las células accesorias. La punta de la neurona sensorial conecta con las células accesorias distales a través de una matriz extracelular de soporte (MEC). En los órganos mecanosensoriales se piensa que esta matriz, conocida como *dendritic cap*, está directamente implicada en el proceso de mecanotransducción al estar unida a los canales iónicos transmitiéndoles la energía mecánica recogida en las estructuras cuticulares. Defectos morfológicos que afectan a la *dendritic cap* afectan a la mecanotransducción, probablemente al interrumpir la transmisión del estímulo.

Hasta hoy día, poco se conoce sobre la composición molecular de la *dendritic cap*. Estudios previos han mostrado que NompA, una proteína transmembrana con un segmento extracelular con un dominio *zona pellucida* (ZP) característico de los componentes formadores de fibrillas en distintas matrices extracelulares, forma parte del andamiaje que la constituye (Chung et al. 2001). Se desconoce cuáles son las moléculas que interaccionan con NompA en la membrana neuronal o cómo la *dendritic*

cap se conecta con las estructuras cuticulares que detectan el estímulo sensorial.

En nuestro trabajo describimos cómo Artichoke, una proteína con repeticiones ricas en leucina (LRR) características de las moléculas de adhesión, está presente en todos los órganos ciliados embrionarios de *Drosophila*, tanto mecano- como quimiosensoriales, y se localiza en la *dendritic cap*. ATK es secretada por una célula accesoria en cada órgano sensorial, pero variando la naturaleza de la misma en los distintos tipos de órganos, lo que implica una regulación específica de su expresión en cada uno de ellos. Nuestro estudio morfológico en embriones mutantes para *artichoke* muestra que las neuronas ciliadas de los órganos mecanorreceptores no se encuentran correctamente estiradas. Para analizar las implicaciones funcionales de estos defectos anatómicos llevamos a cabo estudios del comportamiento locomotor y gustativo larvario. Nuestros resultados muestran que ambos tipos de modalidades sensoriales se encuentran afectadas en individuos mutantes para *atk*, lo que implica que un correcto acoplamiento del cilio sensorial a las estructuras cuticulares es necesario para que los órganos sensoriales sean plenamente funcionales, independientemente de la naturaleza del estímulo. Demostramos que las células accesorias juegan un papel fundamental en la organización y función de los órganos sensoriales.

Introduction

1. INTRODUCTION

Sensory organs are fundamental structures that allow organisms to sense the outside world. Without them it would be impossible to survive in our planet where the endless changing conditions force organisms to constantly adapt their physiology to survive. Sensory organs are biological dictionaries that translate the environmental stimuli into a terminology that the nervous system understands, an electrical jargon. Even those organisms that lack a central nervous system to control their physiology, such as bacteria or plants, possess sensory structures. It is possible to survive without a central nervous system, but not without sensory detectors.

Within the sensory universe, cilia play an outstanding role. Their elongated structure that houses the axoneme, a radially symmetric cytoskeleton of nine microtubules and associated structures, enables them to concentrate the molecular machinery required for sensory transduction increasing its efficiency. However, cilia do not play alone. Most of them are surrounded by accessory structures that provide the physiological context for the transduction process to occur. Mutations in these accessory structures impair sensory transduction as much as defects in the cilium itself do. However, up to date little is known about their molecular composition or

the nature of their interactions with the cilia. In this work we have tried to unravel part of this question by studying how Artichoke, a novel secreted adhesion molecule, is required for providing support in both mechano- and chemosensory cilia in *Drosophila melanogaster*.

1.1 Sensory organs in *Drosophila melanogaster*

Sensory organs in *Drosophila* fall into two main types (Fig.1 and 2). Type I organs, also known as sensilla, are multicellular and each comprises one to four sensory neurons and three to four individually specialized accessory cells. Each type I neuron has a single dendrite with a modified cilium at its distal tip. The surrounding accessory cells enwrap the sensory process and construct accessory structures that distinguish the different sensilla and serve as the stimulus-receiving apparatus (Hartenstein 1988). In contrast, type II organs consist of single, non-ciliated multidendritic (md) neuron with dendritic morphologies ranging from a simple T-shape to extensive arborizations (Affolter, Basler 2007; Kernan 2007).

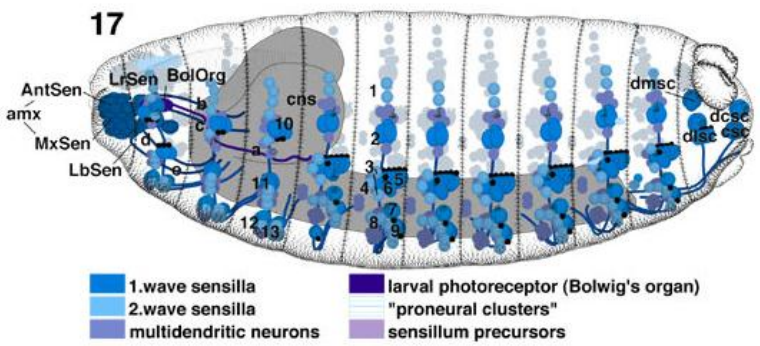

Figure 1: Illustration of the peripheral nervous system of a stage 17 embryo. The whole body of the embryo is covered with mechano- and chemosensilla that will madurate during the late stages of the embryogenesis to be fully functional at the larval stages. The chemosensory machinery is located in the embryonic head (AntSen, MxSen, amx). Each body hemisegment comprises a set of mechanosensory organs that includes both type I mechanosensory organs and multidendritic neurons. Abbreviations: amx, antennomaxillary complex; cns, central nervous system; csc, dcsc, dlsc and dmsc, different types of caudal sensory cones; **1-4, 7, 8, 11, 12**, external sensory organs; **5, 6, 9, 10**, chordotonal organs; **13** Keilin´s organ; (*b*) labral nerve; (*c*) antennal nerve; (*d*) maxillary nerve; (*e*) labial nerve. Image obtained from Flybase.

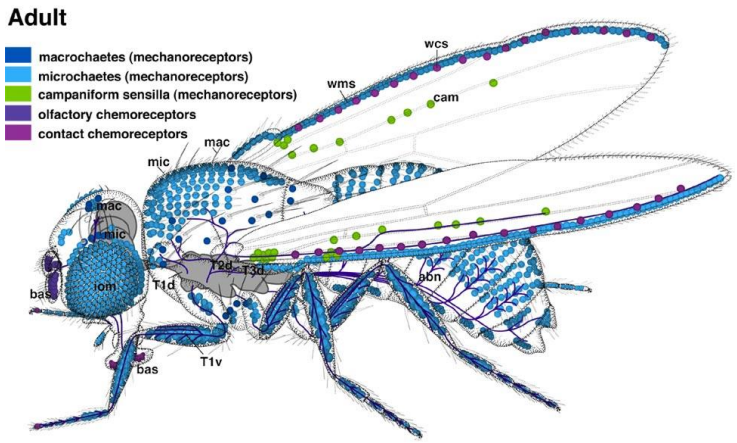

Figure 2: Illustration of the peripheral nervous system of an adult fly. The main sensory structures are the antenna, with the auditory Johnston´s Organ and the chemosensory organs from the third antennal segment; the compound eyes that bear the photoreceptors; the gustatory palps; the campaniform sensilla located along the wings; and the micro- and macrochaetes that cover the whole body of the fly. Abbreviations: abn, abdominal nerves; bas, basiconal sensilla; cns, central nervous system; iom, interommatidial bristles; mic, microchaetae; T1d, T2d, T3d, T1v different nerves of the thoracic segments 1, 2,3; wms, mechanoreceptors of the wing margin. Image obtained from Flybase.

Type I organs can either be chemo- or mechanosensory, depending on the type of stimulus they transduce. Mechanosensory organs can be further subdivided after the organization of the extracellular stimulus-detecting structures. Those sensilla whose detecting structures are external, like a shaft or a cuticular dome, are called **external sensory (es) organs**, and those that lack external structures and are attached to the underside of the cuticle are called **chordotonal (ch) organs** (Hartenstein 1988; Kernan 2007). The most abundant type of **es organs** are the sensory bristles (also

known as chaetae). Each of them is a separately innervated sensory organ. They possess a cuticular curved bristle shaft that serves as the stimulus-detecting structure and is secreted by the trichogen (or shaft) cell and is seated on a socket, the product of the tormogen (or socket) cell (Fig.3B, C). The whole body of the fly is covered by small microchaetae, accompanied by larger macrochaetae that are located in a stereotyped pattern in the head and thorax (Fig.3A). Both types are thought to be tactile-stimulating bristles, although some smaller bristles at legs joints provide as well propioceptive feedback on limb position and locomotion.

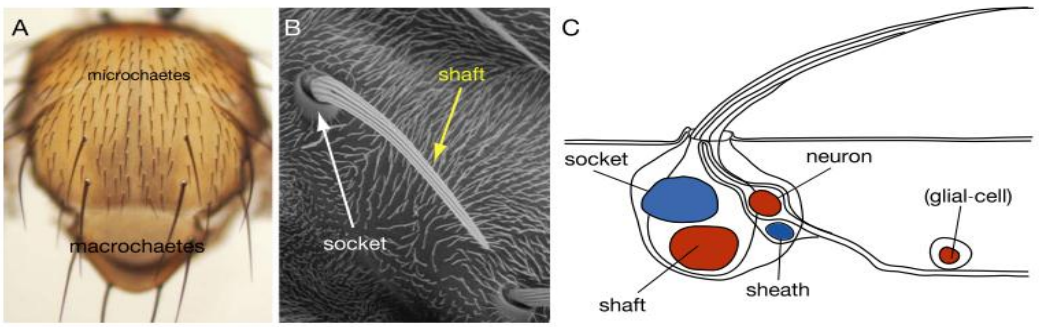

Figure 3: *Drosophila* adult external sensory organs. (A) Photograph showing the dorsal thorax of an adult fly. The thorax is covered by a stereotyped array of larger bristles, known as macrochaetes, and smaller bristles, referred as microchaetes. (B) Scanning electron micrograph showing the external structures of an es organ. A curved bristle shaft secreted by the trichogen or shaft cell emerges from the cuticle and is seated by a socket produced by the tormogen cell. (C) Illustration of a cross section of an es organ showing the cellular organization of the organ. The socket and shaft cells are endoreplicated and their nuclei are bigger than those of the other cells. Red nuclei indicate Notch signaling cells while blue nuclei indicate cells where the Notch pathway is activated during the set of asymmetric divisions that give rise to the organ (Lai, Orgogozo 2004).

Ch organs, in contrast to es organs, lack visible external sensilla and are attached to the underside of the cuticle (Yack 2004). Our study is focused on the analysis of ch organs so a detailed explanation on its structure will be given later. There are prominent ch organs during larval and adult stages. The larval **lateral pentaloscolopidial organ (lch5)** is attached to the body wall in each body hemisegment. It consists of an array of 5 individual ch organs or scolopidia. They develop during the embryonic stages and are located diagonally to the muscles probably to sense their contractions and the relative displacements of the body parts (Klein et al. 2010).

In the **adult**, the main ch organ is the auditory **Johnston´s Organ (JO)** (Fig.4). It is the largest ch organ in the fly and transduces near-field sounds, such as the courtship song (Kernan 2007). The JO is formed by an array of 227 scolopidia (Kamikouchi, Albert & Göpfert 2010) in the second antennal segment (pedicel). Each scolopidium is innervated by 2-3 neurons. The array of scolopidia converges to the joint between the second and third antennal segment (funicle) (a2/a3 joint), where they are attached to the funicular hook (Fig.4B) (Gopfert, Robert 2002). The funicle is also connected to the arista, a receiving structure easily displaced by the air moving mass. Sound-induced movements of the sound-receiver, the stiff entity formed by the arista and the funicle, causes a rotational force on the attachment between a2/a3 (Gopfert, Robert 2002; Eberl, Boekhoff-Falk 2007; Gopfert, Robert 2001). This force is transmitted through the dendritic cap to the ciliated sensory neurons that transduce the signal (Eberl, Boekhoff-Falk 2007).

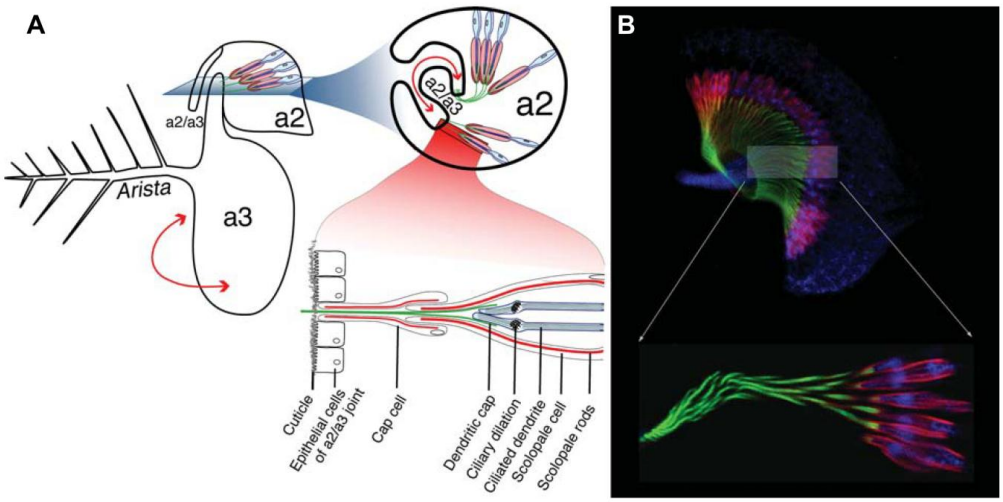

Figure 4: Structure of the Johnston´s organ in *Drosophila*. (A) Schematic representation of the Drosophila antenna and the arrays of sensory scolopidia in the JO. Acoustic stimuli of the arista induce rotation of the a3 relative to the a2/a3 joint and this displacement opens the mechanically gated ion channels. Cross-sectional view through a2 shows the arrangement of the scolopidia that converge at the joint. At the bottom, a detailed view of the cellular composition of individual scolopidia is shown: the dendrite tips are connected to the cuticle via dendritic cap (green) that encloses them. The cap and scolopale cells contain thick actin-based rots (red). **(B)** Confocal image of a pupal antenna expressing the GFP-NompA protein in the dendritic cap (green) and counterstained with Cy3-phalloidin (red) to display the actin rods in the scolopale and cap cells and with anti-horseradish peroxidase to label neurons (blue). A detailed optical section of several scolopidia is shown at the bottom (Eberl, Boekhoff-Falk 2007).

Type I chemosensory organs are further subdivided into **olfactory sensilla**, which detect volatile chemicals, and **gustatory sensilla**, which enable the fly to detect soluble compounds. The adult olfactory sensory system is comprised of two pairwise head appendages, the third segments of the antennae, and the maxillary palps (Fig. 2). These appendages are covered with hundreds of sensory hairs. By contrast, the gustatory system is widely distributed over the animal's body. The main taste organ, the proboscis or

labial palps, is located on the distal end of the head, but flies, like many other insects, have taste sensilla on legs and wings and, in females, on the genitalia (Kernan 2007). The chemosensory equipment of the larval head includes three external sense organs, dorsal organ (DO), terminal organ (TO) and ventral organ (VO), as well as three pharyngeal organs (Fig. 5). Each of these organs consists of several sensilla comprising one to nine neurons and three accessory cells, all of which are collected below a common cuticular hair or terminal pore (Stocker 2008).

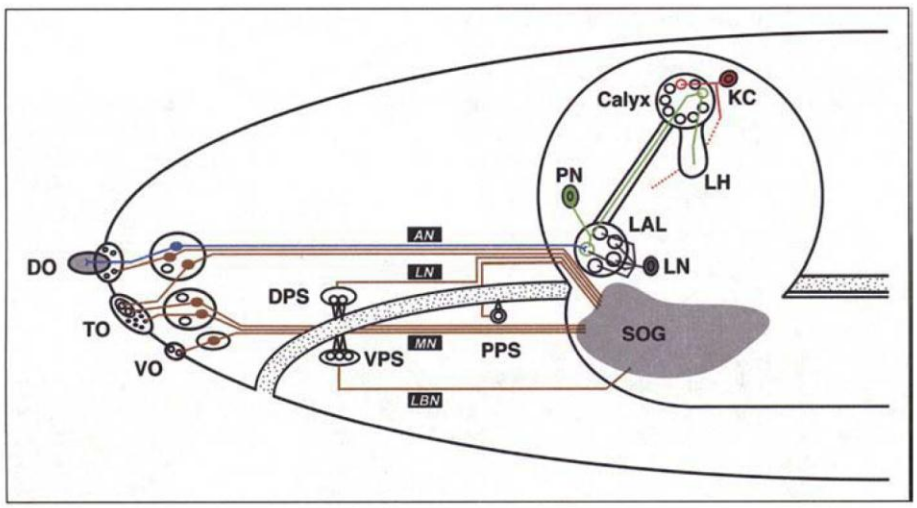

Figure 5: Schematic representation of the chemosensory system of the larval head. The dorsal organ (DO) comprises an olfactory dome (grey) surrounded by 6 peripheral sensilla (small circles). The terminal organ (TO), the ventral organ (VO), as well as the dorsal, ventral and posterior pharyngeal sensory organs (DPS, VPS and PPS, respectively), include mainly taste sensilla. Neuronal cell bodies are located in ganglia basally to each sense organ. The odorant receptor neurons (blue) from the DO send their axon via the antennal nerve (AN) into the larval antennal lobe (LAL). Local interneurons (LN) interconnect the glomeruli of the LAL, while projection neurons (pN; green) link the LAL with the mushroom body calyx and the lateral horn (LH). One of the intrinsic mushroom body Kenyon cells (KC; red) is shown. Taste receptor axons (brown) extend via four different nerves to the CNS and end in the suboesophageal region (SOG). LBN, labial nerve; LN, labral nerve; MN, maxillary nerve (Stocker, 2008).

1.2 Chordotonal organs: a model to study mechanosensory organ assembly

Ch organs are mechanoreceptors that lack any visible cuticular structure and are attached to the underside of the cuticle. They are only present in insects and crustacean and function as proprioreceptors, detecting self-induced movements of limbs and internal organs, or exteroreceptors, detecting gravitational forces or acoustic stimuli (Kernan 2007).

The term ch organ refers to the whole system, which usually comprises one or more special units called scolopidia (Yack 2004). A single scolopididum consists of five cells that are homologous to the cells in other ciliated sensory organs: one to four ciliated sensory neurons; a scolopale cell that envelops the sensory cilium; one or more attachment cells (the cap and the CA cell) associated with the distal region of the cilium and connecting it to the stimulus-detecting cuticular structures, and one or more ligament cells that surround the proximal region of the sensory neuron and anchor it ventrally to the ectoderm by a specialized attachment cell, the ligament attachment (LA) cell (Fig.6A) (Inbal, Volk & Salzberg 2004). Each of these cells is characterized by the expression of a unique set of genes that make it possible to identify each cell by immunodetection methods (Klein et al. 2010).

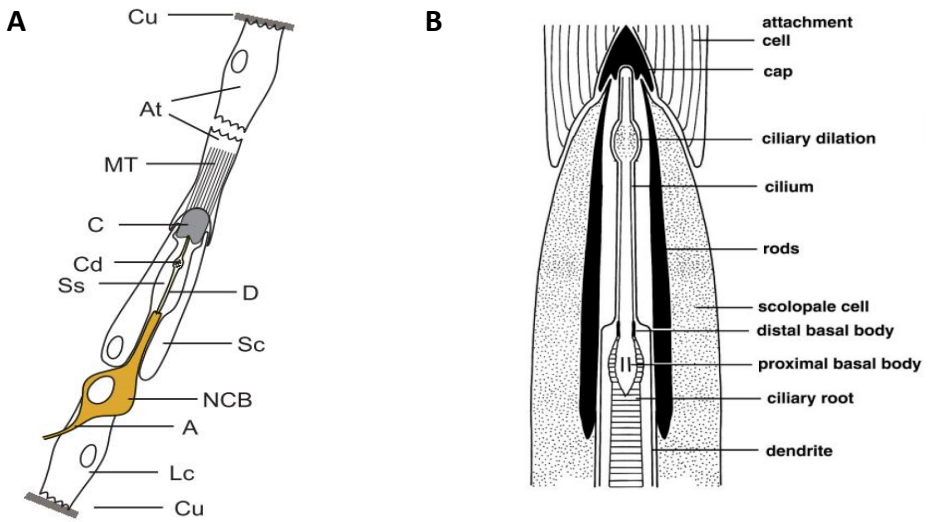

Figure 6: Ch organ morphology. (**A**) Representation of an embryonic ch organ. The sensory neuron dendrite is enwrapped by the scolopale cell that creates around the cilium the receptor lymph cavity. The distal tip of the sensory cilium attaches to the dendritic cap, which is also connecting the cap cell at its distal side. Abbreviations: A, axon; At, attachment cell; C, dendritic cap; Cd, ciliary dilation; Cu, cuticle; D, dendritic outer segment; Lc, ligament cell; MT, microtubules; NCB, nerve cell body; Sc, scolopale cell; SS, scolopale space (Eberl, 2007). (**B**) Detailed view of the cilium and accessory structures. The cilium originates from the basal bodies in the distal region of the dendrite. The electrodense intracellular structure known as the ciliary dilation divides the cilium into distal and proximal region. The cilium is enwrapped by the scolopale cell that creates a sealed lymph cavity for the ion flow. The dendritic cap (cap in the schematic) binds the cilium tip and connects it to the cap cell (Yack 2004).

The cilium of the sensory neuron is the main character in the transduction process. They bear the channels responsible for the sensory transduction. Cilia are distinguished by an axoneme, a radially symmetric cytoskeleton of nine microtubule doublets and associated structures, enclosed in an extension of the plasma membrane. The presence or absence of a central microtubule pair classifies cilia in two types. Those with a central pair (9+2

configuration) usually have a propulsive function, while those without a central microtubule pair (9+0 configuration) are found on many animal cell types, where they are known as “primary” cilia. Sensory cilia are derived from primary cilia and have been modified to varying degrees; most are probably non-motile (Dubruille et al. 2002). In vertebrates, there are many different examples of cilia functioning as sensory structures such as the outer segments of the retinal and pineal photoreceptors, the kinocilia associated with the stereocilia of the hair cells and the multiple cilia on the sensory neurons in the main olfactory epithelium. In *Drosophila* primary cilia are only found in mechano- and chemosensory neurons (Laurençon et al. 2007; Lee et al. 2008).

Sensory cilia are usually associated with a specialized supporting extracellular matrix (ECM) at its distal tip (Emtage et al. 2004). In mechanosensory cilia, this matrix is known as dendritic cap and is especially important since it has been suggested to be directly involved in the transduction process. The very rapid transduction process denotes that channels are directly gated by mechanical stimuli that are probably transmitted through this matrix. The accepted model for the gating of mechanotransduction channels states that channels detect the deflection of an external structure relative to an internal structure (Gillespie, Walker 2001). The ECM acts as a linker between the external structure and the channels and is therefore directly involved in mechanotransduction (Fig.6B). Consistent with this hypothesis, screenings for mechanosensory mutants have always isolated ECM proteins (Chalfie, Au 1989; Chalfie,

Sulston 1981; Kernan, Cowan & Zuker 1994). Such is the case for different mechanosensitive mutant (*mec* genes) in *C.elegans* (Chalfie, Au 1989; Chalfie, Sulston 1981) such as MEC-1 and MEC-5. Although the nature of the interaction between the ECM and the transduction channel is not fully understood, MEC-1 and MEC-5 are reported to be necessary for the localization of the mechanosensory degenerin channel complex and for touch sensitivity (Emtage et al. 2004). In *Drosophila*, only the zona-pellucida (ZP)-domain protein nompA has been so far described to localize to the dendritic cap where it is thought to form the scaffold of the matrix (Chung et al. 2001). *nompA* mutants show both a disorganized dendritic cap with cilia detached from the distal structures and behavioral defects (Chung et al. 2001; Göpfert, Robert 2003), indicating that a proper coupling of the sensory cilia to the cuticular structures is needed for the organ to be functional.

In 2004 Avidor-Reiss et al. carried out a comparative genomic analysis trying to identify candidate genes involved in ciliogenesis (Avidor-Reiss et al. 2004). They found 200 candidate genes conserved in the genomes of ciliated organisms that were absent from the genomes of non-ciliated organisms. Among them a novel LRR protein-encoding gene, the gene *CG5195*, was isolated (Avidor-Reiss et al. 2004). This relationship between *CG5195* and ciliogenesis was confirmed again in a later work from Cachero et al. (2011) (Cachero et al. 2011), where the authors tried to address how proneural transcription factors, apart from its well-studied role in fate specification, trigger the cellular pathways that ultimately lead to the

cilium formation. They focused on mechanosensory cilia by profiling the time course of gene expression that is triggered by the ch organ proneural factor *atonal* at three time points corresponding to the first 3 h of neural development (which would correspond to embryonic stages 11-12). Their results showed how many genes known to be involved in ciliogenesis are directly regulated by *ato*, even at surprisingly early stages of neural development. *CG5195* also appeared as an ATO target. They also find evidence that *CG5195* is regulated by Regulator factor X (RFX*),* a well-known highly conserved ciliogenesis transcriptional regulator, even though *CG5195* is not flanked by a X-box, where RFX binds to activate transcription (Cachero et al. 2011; Durand et al. 2000).

1.3 Leucine Rich Repeat (LRR) proteins

The protein CG5195, that we named Artichoke (ATK), encodes for a novel LRR protein. LRR domains are one of the most common repeated domains across species. They are protein-ligand interaction motifs of 20-30 amino acids length enriched in leucines and are found in a large number of proteins of diverse structure, localization and function in bacteria, fungi, plants and animals (Kobe, Kajava 2001). Many of them have well-known functions in the innate immune system. Many others, especially those with extracellular LRRs (eLRRs), are involved in various aspects of the nervous system development and have been linked to human neurological and psychiatric disorders (Dolan et al.2007).

The N-terminal part of the repeat consists of a conserved 11-residue sequence rich in leucines at defined positions (LxxLxLxxN/CxL, where x is any amino acid, N asparagine, C Cysteine and L positions can also be occupied by valine, isoleucine and phenylalanine). Individual LRRs are arrayed in tandems that together constitute the LRR domain. LRRs possess a curved structure and an exposed β-sheet on the concave side that forms a large binding surface, which makes the LRR domain a very effective protein-binding motif (Kobe, Kajava 2001) and generates a versatile and highly evolvable framework for the binding of diverse proteins and non-protein ligands.

In *Drosophila* many members have been described to account for different aspects of neural development, such as axon guidance, dendrite arborization, target selection, synapse formation and stabilization of connections. Some examples are the glycoprotein Chaoptin, involved in microvillar organization in developing rhabdomeres (Vanvactor et al. 1988) or the proteins Capricious (Caps) and Tartan (Trn) that play important roles in regulating targeting specificity of axons and dendrites in a variety of systems such as the neuromuscular junction (Shishido, Takeichi & Nose 1998) and the visual and olfactory systems (de Wit et al. 2011; Hong et al. 2009). The transmembrane LRR protein LRT acts non-autonomously to better target the muscle and/or arrest its migration upon arrival at its corresponding tendon cell (Wayburn, Volk 2007).

Furthermore, previous studies have shown the importance that eLRR proteins have in the organization of ECM. In *C.elegans*, the transmembrane eLRR proteins LET-4 and EGG-6 and the secreted eLRR protein SYM-1 are required for the organization of the apical ECM in the excretory duct and pore (Mancuso et al. 2012). In mammals, the related eLRR proteins Decorin and LRRTM1-3 influence stromal ECM or synaptic juntion organization, respectively (Kalamajski, Oldberg 2010). In *Drosophila*, the eLRR secreted protein convoluted (conv) is necessary for apical matrix organization during tracheal tube morphogenesis (Swanson et al. 2009).

However, the role of eLRR proteins has not yet been described for the organization of ECM supporting sensory organs. Here we report the identification of Artichoke (ATK) (CG5195), a novel secreted eLRR protein that localizes to the supporting extracellular matrix of all ciliated sensory organs, mechano- and chemosensory in *Drosophila* embryos and at least to the pupal JO. ATK is the first molecule to be described that is secreted into the ECM by the shaft cell in es and chemosensory organs and by the cap cell in ch organs. *atk* mutants show defects in the stretching of the mechanosensory cilia in the lch5 organ, the most important embryonic ch organ, and subsequently locomotion impairments appear in mutant larvae. The colocalization of ATK with NompA in the embryonic chemosensory organs and the chemotaxis defects shown by mutant larvae suggest that an ECM is also present at the tip of chemosensory cilia and that it is also necessary for the chemo-transduction process to occur.

Objectives

2. OBJECTIVES

The aim of this project has been to characterize the expression pattern and function of the novel LRR-protein Artichoke in *Drosophila melanogaster* and to gain insight into the role that the supporting ECM that associates to each sensory cilium tip is playing in different transduction modalities.

In order to do this, we have considered the following specific objectives:

- Description of *atk* expression pattern during the different developmental stages of *Drosophila melanogaster*.
- Generation of *atk* mutant alleles and study of their expression pattern.
- Determination of ATK localization in mechano- and chemosensory organs.
- Analysis of *atk* mutant morphological phenotypes in the embryonic chordotonal organs.
- Analysis of *atk* mutant behavioral phenotypes in larvae and adult. We have considered both mechano- and chemosensory systems to address the behavioral defects.

Materials and Methods

3. MATERIALS AND METHODS

3.1 Fly strains

We used the wild-type line *orizo* and w^{118} to characterize the expression pattern of *atk* and the distribution of ATK protein. To generate *atk* mutant alleles we used the p-element line $Uhg8^{EY07139}$ (Bloomington Stock Centre 20078). For the p-element induced male recombination we additionally used the strain $mwh^2\ ru^1\ kni^{ri\text{-}1}/TM3,\ mwh^2\ ru^1\ Sb^1$ (Bloomington Stock Centre 8852). The $control^{18}$ line, which was generated by P-element precise excision, was used as control line. For the rescue experiments, the P [acman] construct CH322-80A07 was introduced into $y^1\ v^1;\ P\{CaryP\}attP40$ flies according to a standard protocol (Bestgene, CA, USA). To analyze the subcellular localization of ATK, we used the line *GFP-nompA* (Chung et al. 2001) (kindly provided by D. F. Eberl), that expresses GFP in the dendritic caps from ch and es organs.

3.2 Mutagenesis

To generate the mutant alleles, the p-element line $Uhg8^{EY07139}$ was used. The hypomorh alleles atk^8 y atk^{23} were generated by imprecise excision of this p-element and the candidate lines were tested by PCR. The null allele atk^{33} was generated by p-element induced male recombination, after placing by standard meiotic recombination the genetic markers ru^1 y $kni^{ri\text{-}1}$

at both sides of *atk*. PCR was performed to detect genomic deletions in each selected line using the following primers: 5'-AAAGTATTCTAGACCTCAAGGATCAGCAGAAGC-3' (forward primer) and 5'-TATAATGGTACCTATATACGGCGTGTAGACCTTGG-3' (reverse primer). Sequencing was performed to characterize *atk* mutant alleles previously selected by PCR with the primers: 5'-cctgccttcttgatgacaactt-3´(forward primer), 5'-GATTCCTCCGGCTGAAGAG-3'(reverse primer). atk^{33} was sequenced after performing an inverse PCR after digesting the genomic DNA with the enzyme HpaII and using the primers 5'-CCTTTCACTCGCACTTATTG-3'(forward) and 5'-GTGAGACAGCGATATGATTGT-3'.

3.3 *In situ* hybridization

Whole-mount *in situ* hybridization using digoxigenin RNA labeled probes for *atk* was carried out as described (Tautz,Pfeifle, 1989) with minor modifications. A 2,5 kbEcoRI *atk*RNA probe was synthesized using the cDNA clone RE27764. The genotypes examined were wild-type, atk^{8}, atk^{23}, atk^{33} and fly strain $Uhg8^{EY07139}$. Staining was detected using NBT/BCIP reaction (Roche) and viewed using bright-field microscopy.

The Patel et al., (Patel 1994) protocol for combined detection of mRNA and protein in *Drosophila* embryos was used for *atk* antisense RNA probe and mAb22C10, mAb2B10, anti-Prospero, anti-repo, anti-αTub85E double-labeling experiments.

3.4 Antibody generation

Fusion protein containing amino acids 1403-1523 of ATK was generated using genomic DNA as template and the following primer pair: 5'-GCGAATTCAGAGAATCTCCTTGTGCAATCG-3'(forward primer) and 5'-ATGGATCCCCACCAGCTTCTCCTCCACT-3' (reverse primer), containing EcoRI and BamHI restriction sites respectively (underlined sequence). The amplified fragment was digested with the restriction enzymes EcoRI and BamHI, clones in the BamHI- EcoRI site of the gluthatione-S-transferase (GST) gene fusion vector pGEX-2T (Promega) vector and transformed in E.coli BL21 DE3. Selected clones were verified by sequencing.

After induction, the GST-$ATK_{1403-1523}$ protein was purified using the Profinia Protein Purification System (BioRad), and used for antibody generation in guinea pig following conventional procedures.

For immunostaining, tissue samples were stained with the purified anti-ATK antiserum after pre-absorbing with *atk* mutant alleles at 1:250 and 1:500 dilution.

3.5 Antennae Sections

w^{118} flies were anesthetized in CO_2 and kept in ice to maintain them slept. Flies´ heads were removed from their bodies and fixated for one hour in 4% paraformaldehide. After fixation, heads were washed with 1 ml methanol for 5 minutes and 3-5 times with PBS to completely remove methanol. Albumin-gelatine was heated in a water-bath to 45ºC. Silicon moulds for the embedding were also preheated. Albumin-gelatine was carefully poured into the moulds avoiding formation of air bubbles. 4 to 5

heads were put inside albumin gelatin, in a way that the antennae were placed frontally. The moulds were then chilled for 5 minutes at 4ºC, followed by 5 minutes in methanol at room temperature. 30 µm sections were made using Leica vibratome.

3.6 Immunohistochemistry

To study the morphology of the lch5 organ from late embryos, we collected eggs laid for 3 hours (at 25°C, or at 26°C for experiments using GAL4 driver lines) and let them grow until the stage needed, which was verified based on the morphology of the midgut (Hartenstein, Campos-Ortega 1985). Staining of whole-mount embryos was performed using standard techniques (Patel 1994) with minor modifications. The following primary antibodies were used: monoclonal antibody (mAb) 22C10 (anti-Futsch), anti-HRP, (mAb) 2B10 (anti-cut), anti-Prospero (Vaessin et al. 1991), mAb 8D12 (anti-repo) (1:20, all supplied by the Developmental Studies Hybridoma Bank), anti-αTub85E (Matthews, Miller, Kaufman, 1990) (1:10, kindly provided by A.Salzberg) and anti-Supressor of Hairless (Santa Cruz Primary Antibodies, kindly provided by) and mouse anti-GFP (1:100, Roche). Secondary antibodies used were Alexa 488 donkey anti-rabbit and Alexa 555 donkey anti-mouse (1:500 both from Invitrogen). Embryos were mounted in Vectashield (Vector Laboratories) and examined using Zeiss Confocal LSM710 vertical confocal microscope. Z-series were projected to obtain cross-sectional views using ImageJ v1.42 (NIH) software. The secondary antibodies for non-fluorescent staining were biotinylated anti-rabbit and goat anti-mouse (1:500, Invitrogen) detected with Vecta-Stain

Elite ABC-HRP kit (Vector Laboratories). Embryos were mounted in Spurr resin (Sigma).

3.7 Generation of UAS transgenic lines

The cDNA of the *atk* gene was obtained from the cDNA clone RE27764 (Berkeley *Drosophila* Genome Project). Two mistakes in its sequence located in positions 1878 and 4089 had to be repaired using the following primers: 5'-AAAATATTCTAGACTAGGGTGGACTCCCAGTTG-3' (forward primer) and 5'-TATAATGGTACCTTCGATTCTGGCTGAGATCC-3' (reverse primer) for the first mistake; 5'-CCTCAAGGATCAGCAGAAGC-3' (forward primer) and 5'-tctagccacgtgctaaatcg-3' (reverse primer) for the second one. After repairing these two errors the insert was cloned into the pUAST vector. Individual constructs were introduced into *yw* flies by germline transformation according to a standard protocol (Bestgene, CA, USA). Its validity was texted performing *in situ* hybridization in *P013-Gal4>UAS-atk* flies.

3.8 Larval Crawling Analysis

Wandering third instar larvae were placed individually on the centre of a layer of a 1% agarose in a 135 mm Petri dish (diameter). Larval movement was recorded over a period of 100 seconds in an environment room maintained at 25ºC. Path lengths were obtained from traces using NIH ImageJ. Larvae tested were from the stocks *orizo*, $control^{18}$, atk^{23}, atk^{33} and $Uhg8^{EY07139}$. Digital Video movies were captured with Moticam 350 and digitalized with Motic Images 2000 1.2 at 15 frames persecond (fps).

3.9 Dynamic Image Analysis

ImageJ was used for the processing of the digitalized images. The high contrast digital movie frames were analyzed using the ImageJ plugin Mtrack2 that generates a coordinate matrix that contains the centroid positions of the larvae throughout time. These data were used to calculate the different variables using Excel (Microsoft Office). Direction changes were calculated studying the difference between the two angles formed by the three vectors binding four consecutive time points and considering this as "direction change" when this difference was bigger than 30°. The path length was calculated adding all the cartesian distances between two larva position coordinates for each consecutive frames. Speed was calculated dividing the total path length by the time period when the larva was moving (100 seconds of experiment length minus the time period when the larva was not moving). The straight stretch time was calculated adding the time spans when the larva did not change its direction for periods longer than 2,5 seconds.

3.10 Gustatory tests

Gustatory choice tests were performed as described in Heimbeck et al. (1999) (Heimbeck et al. 1999) with minor modifications. A small Petri dish (diameter 35 mm) was placed inside a bigger Petri dish (diameter 85 mm). The smaller Petri dish was filled with 1% agarose and 1M sucrose and the bigger Petri dish was filled with 1% agarose and water. To avoid diffusion, plates were poured immediately before testing. Fifty third-instar feeding larvae were placed on the edge of the smaller Petri dish and allowed to

freely move on the entire surface. They were counted after 5, 15, and 30 min. A Response Index (RI) index was calculated for each time point (RI= N_s - N_c/ N_s+ N_c, N_s and N_c referring to the number present on test and control areas, respectively). Animals found at a distance of 0,5 cm from each side of the plastic bridge were not counted.

3.11 Statistical Analysis

We tested the normality of all our variables with the Kolmogorov–Smirnov test. None of the variables obtained from phenotypical analyses of ch organs follow a normal distribution. Hence, we performed non parametric Kruskall-Wallis Test with these data, followed by the proper procedure to determine which pairs tend to be different (Conover, 1999). Three of the variables obtained in the larval crawling analysis are normally distributed (direction changes, average path and average speed), and the square root of the two other (movement absence and straight stretch) are also normally distributed. To analyze these results we employed ANOVA followed by Bonferroni post-hoc test. Finally the gustatory choice data are analyzed with student t test, as they fit the assumption of normality. All statistical analyses were performed employing SPSS13.

3.12 Recording free fluctuations of the *Drosophila* sound receiver

Measurements were performed as described in Albert *et al*., 2006 and Albert *et al*., 2007 (Albert, Nadrowksi & Goepfert 2007; Albert, Nadrowski

& Gopfert 2007). Flies were anesthetized with CO_2 and affixed with their head, pedicels, wings, legs and halters being stabilized by wax on top of a plastic rod to minimize movements. All measurements were carried out at room temperature (22˚-24˚C) on a vibration isolation table. The displacement of the fly´s antennal receiver was monitored with a computer-controlled Polytec PSV-400 scanning laser Doppler vibrometer (LDV) (Polytec GmbH, Waldbronn, Germany) equipped with a close-up unit and a DD-5000 displacement decoder. The rod holding the fly was placed at focal distance (70 mm) from the LDV, with the arista perpendicular to the laser beam. The laser was focused on the arista tip with the scanning unit of the laser head. The position of the laser spot was monitored online with the coaxial video capture system of the LDV. Only one receiver was examined per fly.

Free fluctuations are used to screen quickly for auditory phenotypes. The *Drosophila* ear amplifies small arista displacements of the JO neurons according to the sound receiver best frequency. Thermal bombardment of air particles are sufficient to displace the sound receiver, in a positive feedback these displacements are amplified by the JO. These active movements have been recorded as free fluctuations (Fig.7).

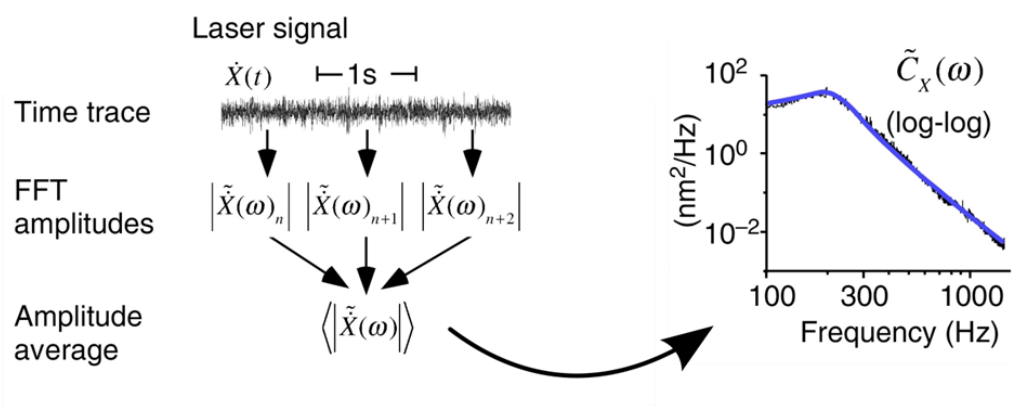

Figure 7: Free fluctuations are recorded with the LDV as a time trace. Recording a 100 s long time trace, dividing it into 1 s long parts and calculating the average fast Fourier transformed signal gives the frequencies making up the sound receiver movements. The fast Fourier transformed date is plotted as the squared displacement / Hz which reflects the activity of moving neurons (Albert, Nadrowski, Göpfert, 2007).

Results

4.1 *atk* is expressed by different types of accessory cells in embryonic type I sensory sensilla.

Drosophila artichoke (*atk, CG5195*) was identified as being involved in ciliogenesis, although a direct confirmation was missing (Avidor-Reiss et al. 2004). In *Drosophila*, only mechanosensory and chemosensory neurons belong to this class of ciliated cells. To identify the cells that express this gene we performed RNA *in situ* hybridization to whole embryos. The pattern of expression is consistent with the distribution of type I embryonic sensory organs. In each body segment expression is seen in cells corresponding to the location of both, ch and es mechanosensory organs (Fig.8A), although expression levels in es organs seem to be higher. Also, chemosensory organs of the head and terminal segments show *atk* expression (Fig.8B). No expression was observed in multidendritic type II organs. Transcription begins at stage 15 in the head and terminal organs and lasts until stage 17, when it firstly disappears from the body segments sensilla and finally from the sensilla in the head and terminal organs. Stage 16 embryos show the strongest expression. As these sensory organs are multicellular, to precisely identify the *atk* expressing cells, we immunostained *atk* hybridized embryos with different antibodies. Lack of colocalization of *atk* RNA with MAb 22C10, a neuronal marker, indicates that *atk* should be expressed in the accessory cells (Fig.8D,D',D¨).

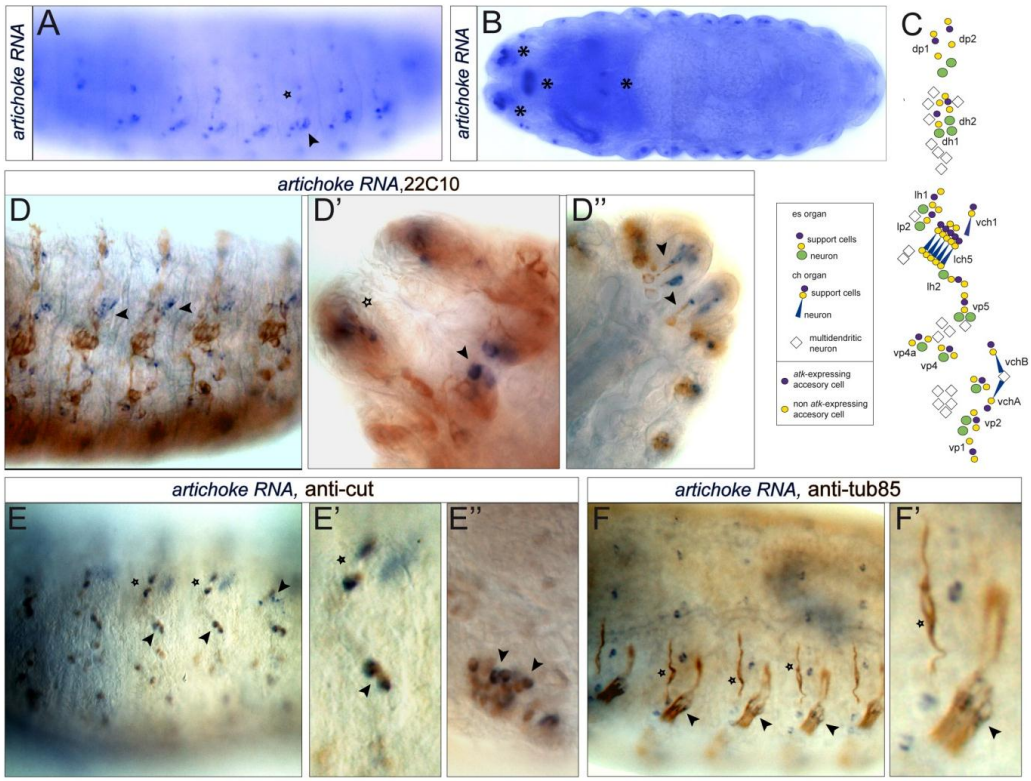

Fig.1. *atk* is expressed by a single accessory cell in each type I sensory organ. (**A-B**) *atk* sense RNA probes were hybridized to wild-type embryos. (A) Lateral view of a stage 16 embryo showing *atk* expression in the peripheral nervous system along the embryonic body segments in ch organs (arrowheads) and es organs (stars). (B) Horizontal view of a stage 16 embryo showing *atk* expression in the embryonic chemosensory organs: dorsal organ (DO), terminal organ (TO), ventral organ (VO), dorsal, ventral and posterior pharyngeal organs (DPS, VPS, PPS respectively). (**C**) Schematic diagram of the sensory organs of a single abdominal hemisegment of a stage 16 embryo showing *atk* expression pattern (purple cells). (**D-F**) *atk in situ* hybridization double inmunostained with different primary antibodies: 22C10 (D), that stains all neurons; anti-cut (E), that stains all the cells in the es organs (although after the *in situ* hybridization treatment the neurons and sheath cells are hardly labeled and only the shaft and socket cell are clearly stained), and anti-tub85E (F), which stains the cap, CA and ligament cells of the ch organs. (D) *atk* is not expressed by the sensory neurons (arrows) in the abdominal mechanosensory organs (D), nor in the chemosensory organs of the head (D') or the posterior spiracles (D''). (E) *atk* is expressed by the shaft cell (stars) in es organs and chemosensory organs (E'') and by the cap cell (arrowheads) in ch organs (F,F''). N: neuron; S: shaft cell; SO: socket cell; CA: cap attachment cell, CAP: cap cell.

Ch organs, the internal mechanosensors, have five individually specialized accessory cells that enclose the sensory process and construct accessory structures. Four of them, the ligament, cap and the two attachment cells, express α-tubulin 85E (α-Tub85E) (Inbal, Volk & Salzberg 2004; Matthews, Miller, Kaufman, 1990). Double staining *atk* RNA and anti-α-Tub85E shows colocalization in one cell type, and the characteristic morphology of this cell and the proximity to the sensory cilium suggest it to be the cap cell (Fig.8F,F') and rules out the possibility of *atk* being expressed by the CA cell. We confirmed this results by immunostaining *atk* hybridized embryos with the ligament cells and scolopale cells specific antibodies anti-Repo and anti-Prospero respectively, which ruled out *atk* being expressed by these cells types (Fig.9B,D).

Es comprise one bipolar neuron and three accessory cells. Again, *atk* is not expressed in the neuron, but in one of the accessory cells. Cell lineage studies have shown that the equivalent of the chordotonal cap cell in the es is the socket cell (Lai, Orgogozo 2004). This cell secretes de receptor lymph, with high potassium content and low calcium, and it is the only one to express the transcription factor Supressor of Hairless (Su(H)) (Barolo et al. 2000). But the socket cell does not express *atk* as demonstrated by the double staining of the hybridized embryos with anti-Su(H) (Fig.9C). To discern between sheath and shaft cells as *atk* expressing accessory cells, we labeled *atk* RNA hybridized embryos with anti-Cut antibody (Fig.8E,E',E''). Actually, all accessory cells in the es organ express the

transcription factor Cut, although with different intensities (Blochlinger et al. 1990).

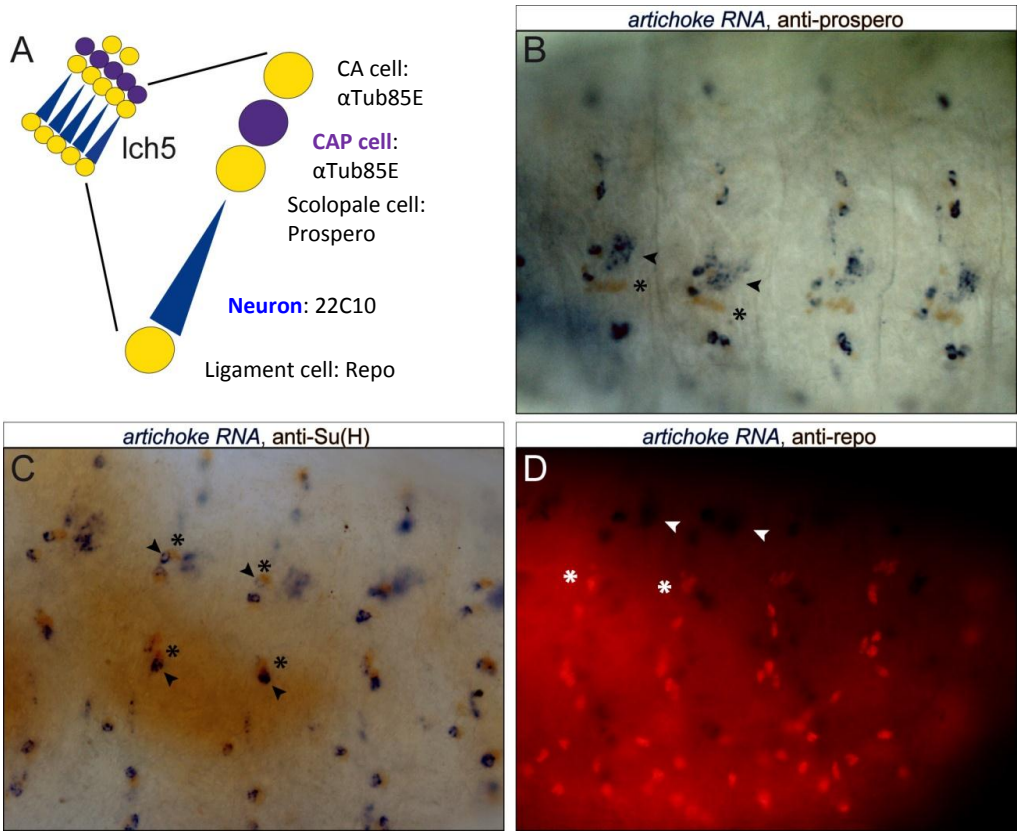

Figure 9: *atk* is only expressed by the cap cell in ch organs and the shaft cell in es organs. **(A)** Schematic picture of the cellular organization of the lch5 organ, showing a detailed draw of a single scolopidia and the arrangement of the ligament, neuron and the scolopale, cap and CA cells. Different genetic markers have been used to confirm the previous results in Fig. 8 that indicate that the *atk* expressing cells are the cap cell in ch organs and the shaft cell in es organs. **(B)** Double staining of *atk* hybridized embryos with the scolopale cell marker anti-prospero. The cells expressing *atk* (arrowheads) are located distally to the scolopale cells (asterisks) in the lch5 organs. **(C)** Double staining of *atk* hybridized embryos with the es organ socket cell marker anti-Su(H). There is not colocalization between both cells types, although *atk* expressing cells (arrowhead) are located adjacent to the socket cells (asterisks). **(D)** Double staining of *atk* hybridized embryos and the ligament cell marker anti-repo. Ligament cells (aterisks) are located at the opposite edge of the lch5 organ compared to the *atk* expressing cells (arrowhead).

This fact helped us to discard the sheath cell as *atk* positive, since in our hands, in immunostained embryos with Cut antibody after the *in situ* hybridization only the cells with high levels of Cut (shaft and socket cells) were detected, and the socket cell has been previously discarded after the immunostaining with anti-Su(H). From these results we can conclude that artichoke is expressed in different types of accessory cells in type I sensory sensilla: the cap cell in the ch organs and the shaft cell in the es organs.

4.2 Generation of *atk* mutant alleles.

atk is located on the third chromosome at 77C3-C4. The P-element $Uhg8^{EY07139}$ inserted 170 bp downstream the 3' end of *atk* was mobilized to generate aberrations affecting the *atk* locus. Three *atk* mutant alleles were generated, two of them hypomorphs and one null. The hypomorph alleles, atk^{8} and atk^{23}, were generated by imprecise excision of the p-element $Uhg8^{EY07139}$. These alleles delete approximately half a kilobase from the 3' region of the gene; atk^{8} deletes 514 bp and atk^{23} deletes 436 bp (Fig.10A). Analyzing the expression profile of *atk* in atk^{8} and atk^{23} mutant flies by *in situ* hybridization shows that *atk* expression is maintained in the chemosensory organs of the embryo head, but it is no longer detectable in the mechanoreceptors from the body segments (Fig 10C). To study the complete loss of function of *atk* a third allele was generated by P-element induced male recombination, atk^{33}. This allele deletes 18 kb downstream the p-element insertion site. *In situ* hybridization of atk^{33} mutant flies reveals no expression of a*tk* in the embryo (Fig 10E).

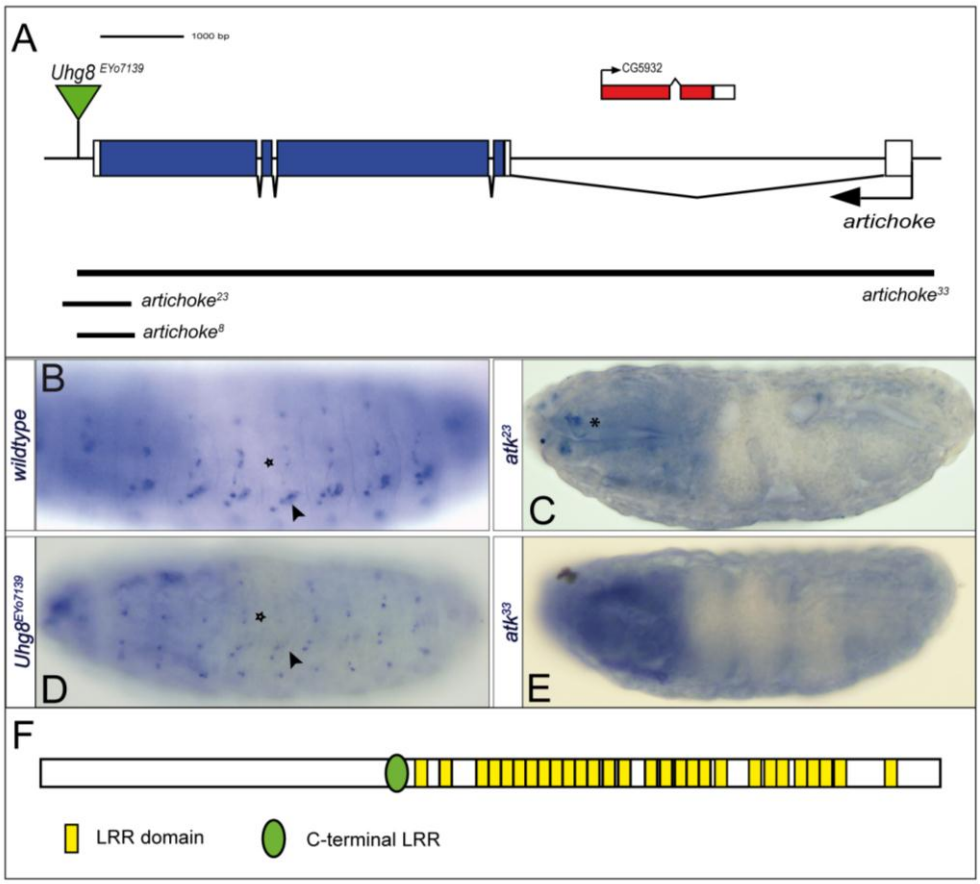

Figure 10: *atk* mutant allele generation. (**A**) Schematic of the *atk* locus. Shown are the intron-exon structure, the location of the p-element $Uhg8^{EY07139}$, the extent and designations of the alleles generated by imprecise excision of the $Uhg8^{EY07139}$ p-element (*artichoke*^{8} and *artichoke*^{23}) and by $Uhg8^{EY07139}$ p-element induced male recombination (*artichoke*^{33}). Also the localization of the gene *CG5932*, which is codified within the first exon of *atk*, is depicted. (**B-D**) Stage 16 embryos hybridized with an *atk* antisense RNA probe: chemosensory organs (asterisk), es organs (stars) and ch organs (arrowheads). (**B**) Wild-type embryo showing *atk* expression in all the ciliated organs of the peripheral nervous sytem. (**C**) In *atk*^{23} mutant embryos, *atk* expression disappears in the body segments, it only persists in the chemosensory organs from the embryo head (asterisk). (**D**) In embryos homozygous for the p-element $Uhg8^{EY07139}$, *atk* expression disappears only from the ch organs (arrowhead), but it persists in the es organs (star). (**E)** The null *atk* null mutant *atk*^{33} shows no *atk* expression. **F**) Predicted Atk protein of 1535 amino acids length.

In addition, *Uhg8*^{EY07139} transgenic flies show as well defects in *atk* expression, since it is no longer detectable by *in situ* hybridization in the embryonic ch organs and seems to diminish in the es organs (Fig 10D). *atk* expression in the chemosensory organs does not appear to be affected.

4.3 ATK localizes to the dendritic cap of type I sensory organs.

The predicted ATK protein contains 1535 amino acids (Fig. 10F). Analysis of the ATK domain structure suggests the presence of 29 LRRs. We generated an antibody against the C-terminal region of ATK (amino acids 1403-1523), which does not bear LRR domains. When used to immunostain embryos, the antisera labeled all type I sensory organs in a dot located adjacent and distally to the tip of the sensory cilium (Fig.11). Although all mechanosensory organs express ATK, es organs show higher levels of protein than ch organs, supporting the *in situ* hybridization results. Double immunostaining with the NompA fusion protein *nompA*-GFP, a transmembrane protein localized to the dendritic cap produced by the scolopale or thecogen cell (Chung et al. 2001), identifies the structure where ATK is expressed as the dendritic cap, an electrodense matrix that binds the tip of the sensory cilia to the stimulus-detecting structures, so that the dendrite is properly stretched to transduce the signal. The antibody also faintly stains the cytoplasm of the cells where ATK is expressed. No signal was associated with either type II sensory organs or any structure outside the PNS.

In ch organs, ATK localizes to the site of connection of the dendrite tip and the cap cell (Fig.11C). In these organs, the dendritic cap contacts the distal third of the cilium, but ATK is localized only to the distal tip of the dendritic cap, in the point of connection to the cap cell. The cap cell is, in turn, connected to the CA cell, an epidermic cell that receives the mechanical stimulus. In es organs, ATK labels a dot at the base of each mechanosensory bristle, at the point where the ciliary dendrite connects the base of the hair (Fig.11D).

Strong staining is as well to be seen in the embryonic chemosensory organs (Fig.12). The larval chemosensory system of *Drosophila melanogaster* includes three external sensory organs: the dorsal organ (DO), the terminal organ (TO) and the ventral organ (VO), as well as three pharyngeal organs. Each of these organs consists of several sensilla comprising one to nine neurons and three accessory cells. While the DO seems to be a mixed organ for taste and smell that ends in a multiporous "dome" suggesting olfactory function surrounded by six peripheral gustatory sensilla, the TO and VO respond to tastants only and are characterized by a terminal pore (Stocker 2008). The three pharyngeal organs include multiple sensilla and include gustatory and probably mechanosensory organs (Singh, 1997).

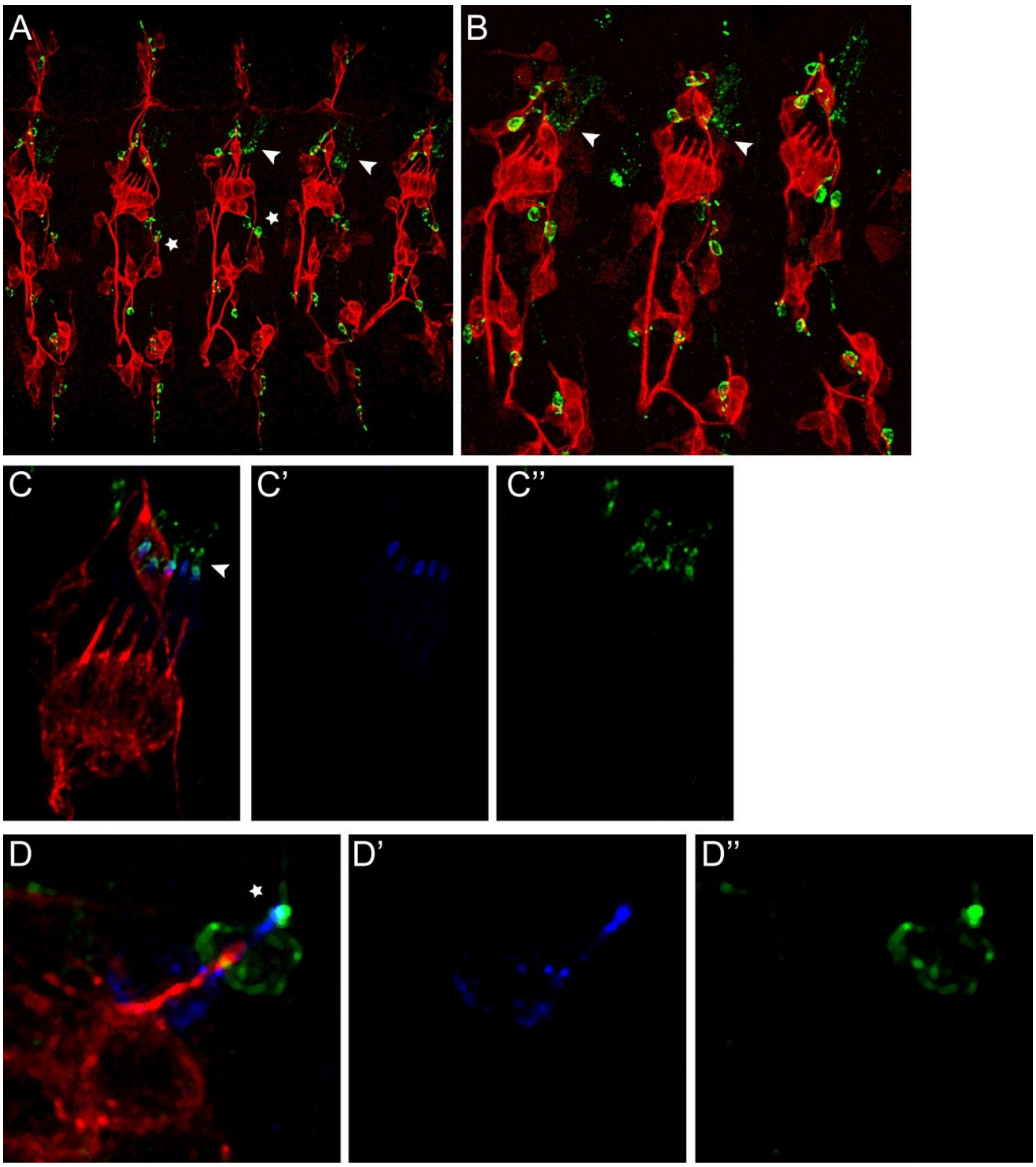

Figure 11: ATK localizes to the distal region of the dendritic cap in embryonic mechanosensory organs. (**A-B**) Embryonic hemisegments immunostained with 22C10 (red) that labels the mechanosensory neurons (up to the inner dendritic segment, but not the cilia) and with anti-ATK. ATK locates to a structure placed apically to the sensory cilia in each type I mechanosensory organ, both in ch (arrowhead) and es (star) organs. Es organs show higher levels of protein. (**C-D**) Detailed view of embryonic mechanosensory organs immunostained with 22C10 (red), anti-GFP (blue) to label the fusion protein NompA-GFP that marks the dendritic cap from ch and es organs, and anti-ATK (green). (**C**) In lch5 organs ATK localizes to the distal region of the dendritic cap (arrowhead), partially overlapping with NompA-GFP but extending its location to form an umbrella-like structure around the latter. (**D**) In es organs ATK also partially overlaps with NompAGFP and extends its location apically (star).

ATK is present in all these organs types (Fig. 12). The anatomical organization of these organs has not yet been described for *Drosophila*, but our results show that it is very similar to the morphology described for the house fly *Musca domestica* (Chu, Axtell 1971; Chuwang, Axtell 1972; Chuwang, Axtell 1972b). In the house fly, the DO possesses a large-fluid containing vacuole that fills the organ from the proximal region where the cellular bodies lie, to the distal lumen of the dome. Microvilli from the trichogen and tormogen cells protrude into the vacuole and are thought to secret compounds into it. The sensory cilia surround the vacuole and branch after reaching the dome, but are not exposed to the exterior, so that to reach their olfactory receptors, the chemicals should cross pores that go through the dome (Chu, Axtell 1971). The distribution of the anti-ATK antibody in the *Drosophila* DO, forming a vacuolar-like elongated structure in the middle of the sensory cilia (Fig. 12D), suggests that this structure is also present in the fruit fly. The expression studies show that it is synthetized by the trichogen cell that secretes it to this central vacuole. On the other hand, in the TO and VO, ATK appears to be part of the ECM that wraps each cilia up to the point where the cilium enters the cuticular pore and exposes itself to the environment (Fig.12C) (Chuwang, Axtell 1972; Chuwang, Axtell 1972b). This is also the case in the gustatory organs from the pharyngeal region (Fig.12B).

Strong expression is also to be seen in the anal sensory cones.

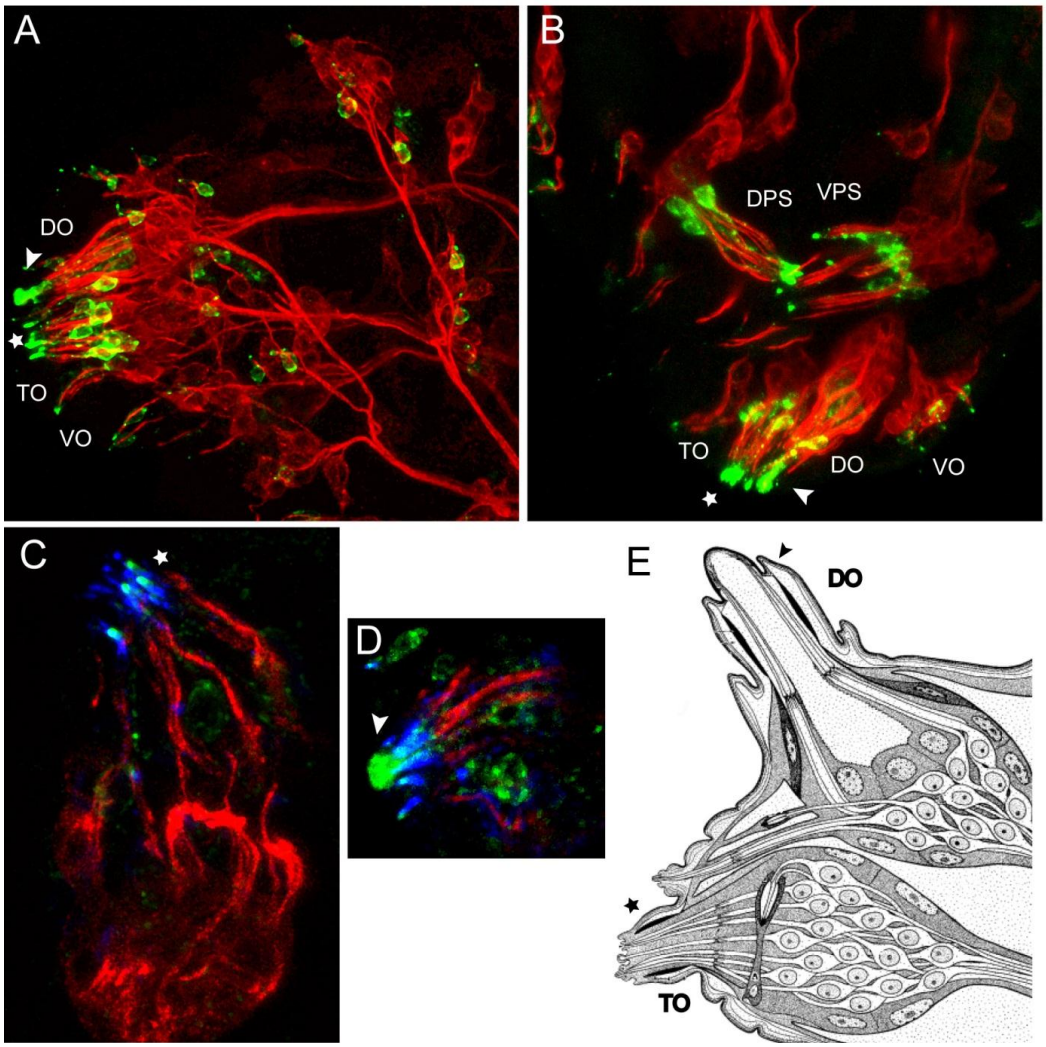

Figure 12: ATK is present in all chemosensory organs from the embryo. (**A-B**) Embryonic chemosensory organs of the embryo head immunostained with the neuronal marker 22C10 (red) and anti- ATK (green). DO: dorsal organ, TO: terminal organ, VO: ventral organ; DPS: dorsal pharyngeal organ; VPS: ventral pharyngeal organ. In the DO (arrowhead) and the TO (star) ATK locates at the tip of the chemosensory neurons. (**C-D**) Detailed view of the TO (**C**) and DO (**D**) organs immunostained with 22C10 (red), anti-GFP (blue) to label NompA-GFP fusion protein and anti-ATK (green). (**C**) In the TO, ATK localizes to the tip of each sensory cilium.(**D**) In the DO, ATK forms a vacuole-like structure in the middle of the cilia and extends distally probably filling the space in the olfactory dome. (**E**) Representation of the dorsal and terminal organ (DO,TO) of the house fly *Musca domestica*, these structures are very similar to those in *Drosophila* (Chuwang, Axtell 1972).

4.4 *atk* mutant embryos show defects in the stretching of the chordotonal organs.

Once identified ATK location to the supporting ECM of type I sensory organs, we analyzed atk mutant embryos for morphological defects. In mechanosensory organs, the dendritic cap is necessary to couple the cilium to the detecting cuticular structures and transmit the mechanical stimuli to the transduction channels. The lateral pentascolopidial (lch5) organ consists of an array of five individual scolopidia clustered together. It is located in the lateral region of each abdominal embryonic hemisegment, where it is anchored at both ends to the cuticle detecting stretch variations. During larval locomotion they provide proprioceptive feedback (Kernan 2007).

The ciliary outer segment of ch organs sensory process is divided into two structurally distinct sections (Kernan, 2007): a proximal connecting cilium with the common 9+0 axoneme, separated by a ciliary dilation from the distal section of the cilium which is filled with an array of densely packed microtubules (Lee et al.2010). To study the morphology of the neurons and cap cells of the lch5 organ, we double stained stage 16 and 17 embryos with both (mAb) 22C10, that stains all sensory neurons up to the ciliary dilation, but does not mark to the cilium tip, and anti-αTub85E (Matthews et al. 1990).

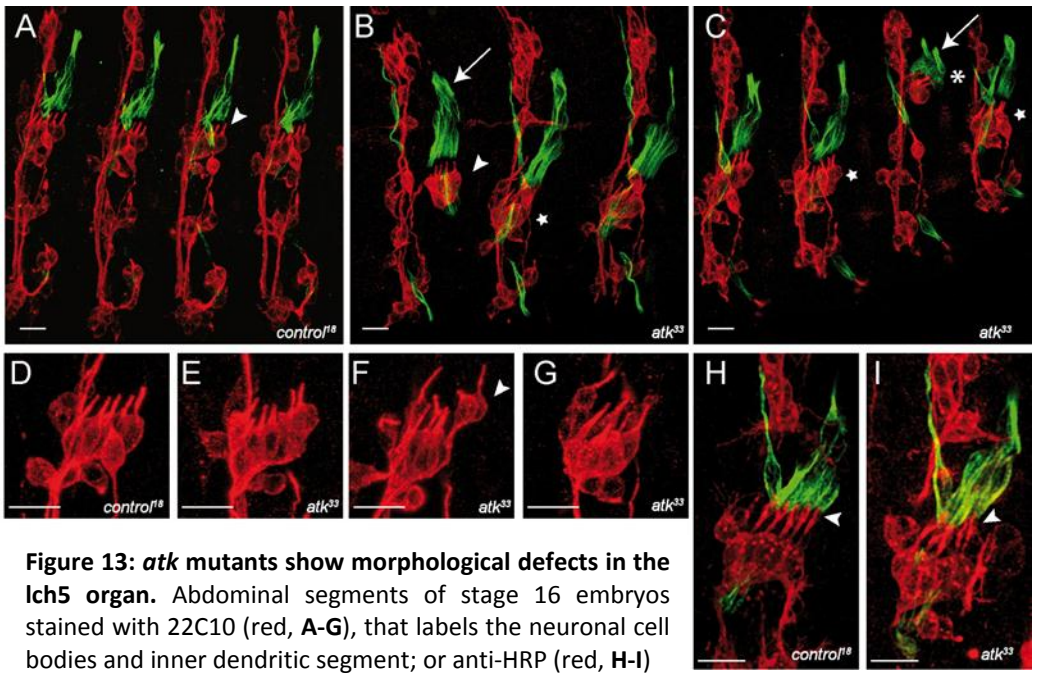

Figure 13: *atk* mutants show morphological defects in the lch5 organ. Abdominal segments of stage 16 embryos stained with 22C10 (red, **A-G**), that labels the neuronal cell bodies and inner dendritic segment; or anti-HRP (red, **H-I**) that additionally labels the ciliary outer dendritic segment, and anti-αTub85 (green), that labels all accessory cells from ch organs except the scolopale cell. (**A-C**) Images of stage 16 embryonic abdominal hemi-segments A2-A5 with the genotypes indicated. (A) Image of a *control*^{18} embryo. The sensory dendrites are organized and parallel to each other (arrowhead) and the cell bodies are located in a linear row within each organ. (B-C) Images of *atk* mutant embryos. Some organs are separated from the nerves (arrowhead), while others are dorsally displaced (asterisk). Moreover, some lch5 organs show an "artichoke-like" phenotype (stars), with both dendrites that are not parallel and disorganized cell bodies. (**D-G**) Detailed view of the sensory neurons of individual lch5 organs from wild-type (D) and *atk* mutant embryos (E-G) showing different phenotypes: "artichoke-like" organ with non-parallel dendrites and disorganized cellular bodies (E); *atk* mutant lch5 organ where two neuronal bodies are separated from the rest of the organ (F; arrowhead) and *atk* mutant lch5 organ where the cell bodies superimpose each other (G). (**H-I**) The cilium seems to contact the dendritic cap in *atk* mutant embryos (I; arrowhead) in a similar way to wild-type individuals (H; arrowhead).

The 5 scolopidia of lch5 wild-type organs are arranged in a row with their cilia pointing to the dorsal posterior region of the embryo (Fig.13A,H). These cilia are parallel to each other and keep the same distance between them. Their tips penetrate the cap cells and contact them through the

dendritic cap. *atk* mutant embryos show defects in the morphology of lch5 organs (Fig. 13B,C,E-G). *atk* mutant dendrites are not parallel, but pointing out to different places in the space (Fig.13B,C,E). *atk* mutant neuronal cell bodies are as well disorganized. This disorganization implies that in different *atk* mutant alleles, an average of 86,5 % of the cell bodies (n=87) are dorso-ventrally (Fig.13B,C,D) or laterally disarranged or superimposed (Fig.13B,C,G). Occasionally the two most distal cell bodies in the lch5 organ (Fig.13F) or even the whole organ (Fig.13B,C) are dorsally shifted. These phenotypes remind us of the rounded shape an artichoke, what gives the gene its name. We performed a morphological score of these phenotypic traces blind to the genotype of the embryo on each slide among different *atk* alleles. The results of this analysis are summarized in Table 1. We considered a lch5 organ to be 'artichoke-like' when both the orientation of its dendrites and the arrangement of its cellular bodies are defective. The morphological deformation is likely to be a consequence of an insufficient tension between the sensory cilium and the cap cell, probably due to a faulty composition of the dendritic cap that results in a defective orientation of the organ. Staining mutant embryos with anti-HRP, a neuronal marker that stains the cilia up to the tip (by contrast 22C10 only stains up to the ciliary dilation), shows that the *atk* mutant cilia contact the dendritic cap, confirming that the morphological defects observed should be of tension and not of disconnection (Fig.13H).

The overexpression of *atk* in a wild-type genetic background does not yield a significant phenotype in the lch5 organ (data not shown).

Table 1. Morphological defects in *atk* mutants

	n	Total neurons (up to 35)	% defective dendrites	% disorganized neuronal bodies	% "artichoke-like"
Control[18]	20	34.6± 0.7	21± 22	39± 23	11± 14
atk[8]	25	31.9± 2.1***	52± 21***	77± 21***	42± 21***
atk[23]	22	32.8± 1.5***	85± 18***	94± 11***	73± 22***
atk[33]	25	32.3± 1.4***	71± 25***	86± 18***	55± 27***
Uhg8[EY07139]	15	32.4± 2.2***	74± 25***	89± 14***	68± 28***

Embryos were stained with 22C10 to label the sensory neurons. Different parameters concerning neuron number and morphology were scored blind with respect to the genotype of embryos on each slide. Whole lch5 organs were examined for defects on the number of neurons (indicated as the total number of neurons from the seven lch5, therefore a maximum of 35), the percentage of lch5 organs with defective dendrites or disorganized neuronal bodies, and the percentage of organs showing both deficiencies,

4.5 *atk* mutant larvae exhibit locomotor defects

Many ch organ mutants show defects in larval locomotion. Although the muscle contractions that direct larval locomotion are controlled by the CNS, a PNS feedback is required to elicit a coordinated larval locomotion. Ch organs are fundamental part of this sensory machinery (Caldwell et al. 2003). To study whether *atk* mutants show locomotion defects as a consequence of the morphological defects observed in the lch5 organ, we performed a larval crawling assay on third instar larvae. We accomplished image analysis to determine the specific nature of the defects affecting locomotion in *atk* mutants, including number of direction changes (Fig. 14A); average path, (Fig. 14B); average speed (Fig. 14C) and average

straight stretch period (Fig. 14D) (See Materials and Methods for calculations details). Our results show that all *atk* mutants exhibit severe defects in larval locomotion (among *atk* hypomorph mutants only atk^{23} individuals were studied, as the deletion lenght is almost the same for atk^{23} and atk^{8} mutants). There was a significant increase in the number of direction changes for all *atk* alleles studied (Fig. 14A), as well as a significant reduction of the total path length (Fig. 14B) and of the average speed (Fig. 14C). Furthermore, the duration of linear locomotion between episodes of turning was reduced in all *atk* mutants (Fig. 14D). Control larvae exhibit more regular and persistent linear locomotion, with fewer and shorter intervals of decision-making and turning, whereas *atk* mutant larvae take a longer time to decide which way to follow, staying on the same point and turning their bodies several times in different directions. Linear locomotion paths were scarce and very short.

On the other hand, $Uhg8^{EY07139}$ homozigous transgenic flies showed a very similar phenotype to the other *atk* mutants in all the variables studied, although concerning the variables 'average speed' and 'path length' the differences with the controls were less significant (Fig. 14B-C). This could be due to the fact that although these individuals show no detectable expression of *atk* in ch organs by *in situ* hybridization, they retain a detectable expression in the embryonic es organs that could account for the intermediate phenotype between wild type and *atk* mutant individuals.

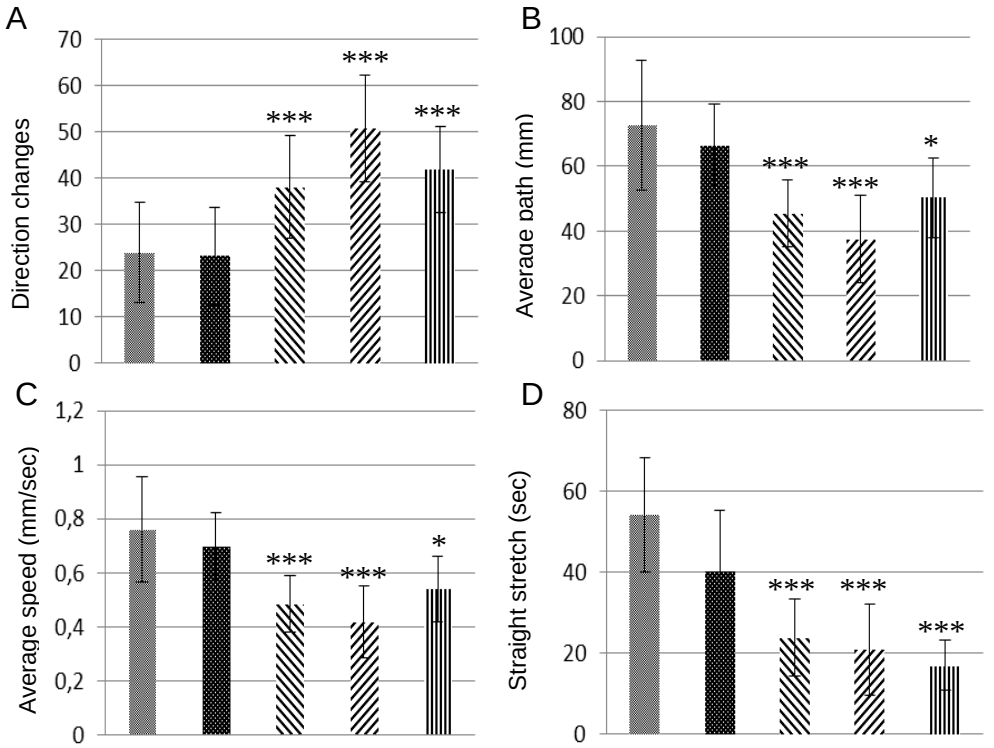

Figure 14: *atk* mutants show larval locomotion defects. Shown is the mean for different parameters describing larval locomotion with standard deviation bars. All *atk* mutants (atk^{23},atk^{33} and $Uhg8^{EY07139}$) show defects in larval locomotion compared to wild-type individuals (*orizo* and $control^{18}$) as showed in the diagram that described different locomotion parameters: (**A**) direction changes, (**B**) average path (mm), (**C**) average speed (mm/second) and (**D**) straight stretch onds) . Significant difference was established by Bonferroni Test. < 0.05; **, P < 0.01; ***,P < 0.005.

4.6 *atk* mutant larvae show a reduced gustatory response to sucrose

ATK is present in each gustatory organ from *Drosophila* embryos (Fig.12). It localizes to the matrix surrounding the cilium from the ciliary dilation to the base of the pore where the tip of the cilium is exposed to the exterior environment to detect the tastants. The function of these organs does not rely on the tension between the stimulus-detecting structures and the sensory dendrite to transmit the mechanical force, as it does in mechanosensory organs, but an inappropriate conformation of the structure that protects and stabilize the cilium may impinge the stimulus transduction, too.

To elucidate ATK physiological function in chemosensory organs we performed gustatory tests on $control^{18}$ and atk^{33} mutant feeding larvae. Insects can distinguish between the principal tastants sweet, sour, salty and bitter (Singh, 1997). Sugars always elicit an attractive response in flies (Miyakawa 1982). To assess larval behavior, we used a simple choice test between 1% agarose and 1% agarose + 1M sucrose on agarose Petri dishes (Fig.15A). Feeding larvae were place on the edge between both surfaces and counted after 5 min.; 15 min. and 30 min. Our results show that atk^{33} mutant larvae show a significantly reduced response to sucrose compared to $control^{18}$ larvae (Fig.15B).

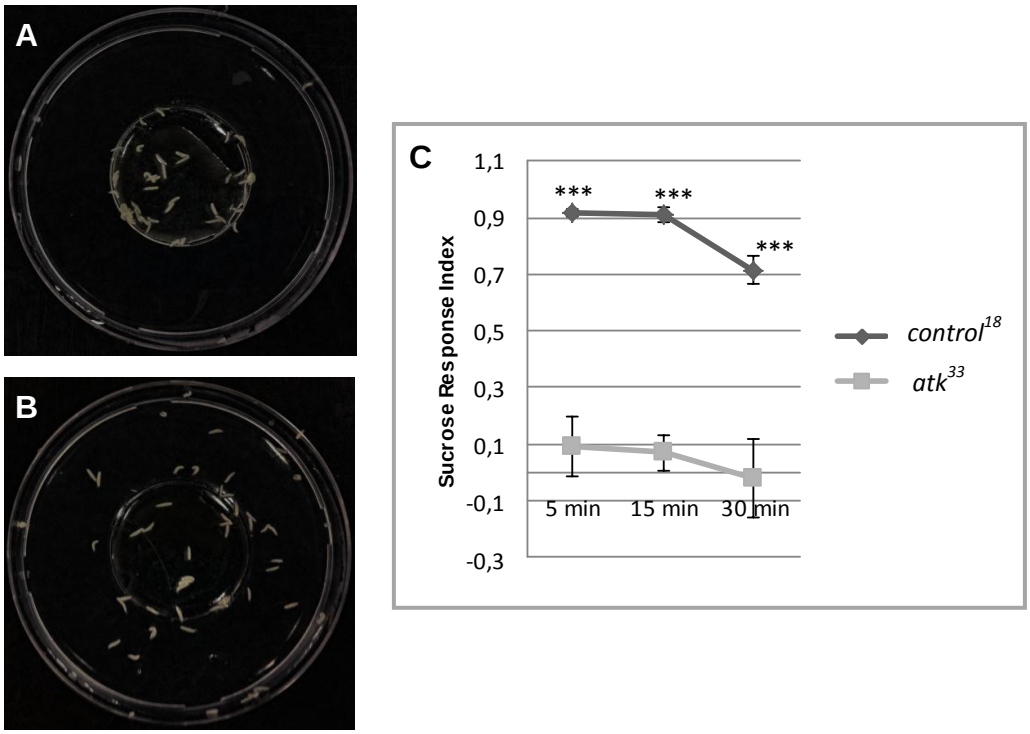

Figure 15: *atk* mutants show a significantly reduced response to sucrose. (**A,B**) Gustatory test plate assay. A small Petri dish with 1M sucrose in 1% agarose was placed inside a bigger Petri dish filled with 1% agarose. Fifty larvae were placed on the edge between both Petri dishes. Most *control*^{18} larvae stay on the sweet surface (**A**), while *atk*^{33} larvae move on top of both dishes (**B**). (**C**) The behavior of *control*^{18} and *atk*^{33} feeding larvae was monitored over 30 min. Animals were counted at the indicated time. The RI (Response Index), that was calculated as explained in Material and Methods, is represented. Error bars indicate SEM. Significant difference between both phenotypes was calculated by Mann-Whitney Test. ***, P < 0,001.

4.7 *atk* expression in the pupae and adult Johnston´s Organ

The larval peripheral nervous system degenerates during the pupal stages when a new adult peripheral nervous system emerges. Therefore, we wanted to investigate whether ATK was also playing a role in the assembly of adult sensory organs.

The JO is the main ch organ from adult flies. It is composed of 227 individual scolopidia and is situated between the second and the third antennal segment. To check for *atk* expression, we performed anti-ATK immunohistochemistry in adult (Fig.16) and pupal JO (Fig.17). Our results rule out the presence of ATK in the adult JO (Fig.16). This agrees with the pattern of *atk* expression in the embryo, in which we find *atk* expression during the late embryonic development of the sensory organs, but the expression fades in the mature larval organs.

Concerning pupae, our results indicate that there is some expression of *atk*, although the protein levels are low (Fig.17). The protein is present in WPP (white pre-pupal) + 3days pupae, but not in later ones. We could not perform analysis on earlier pupae since the tissue falls apart.

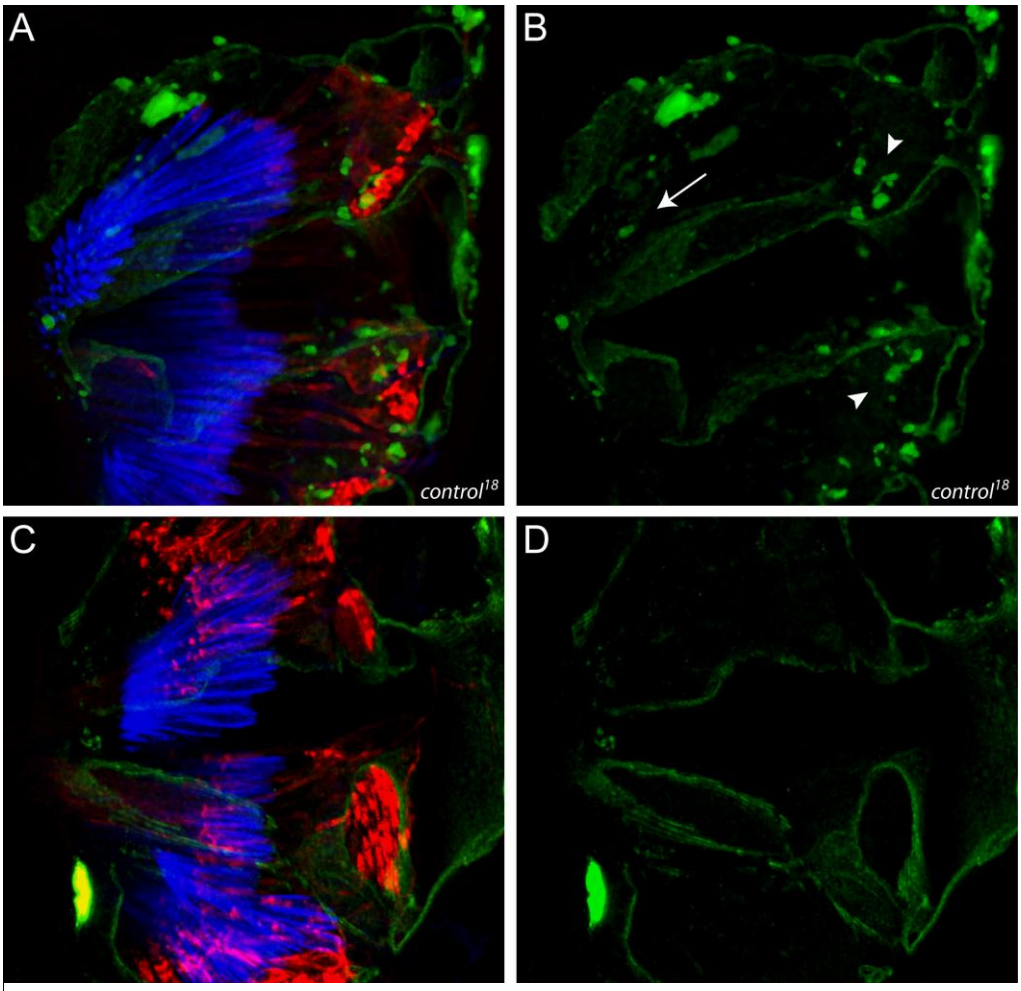

Figure 16: **Atk is not present in the adult JO.** Adult JO immunostained with 22C10 (red) anti-Atk (green) and phalloidin (blue) that stains the actin rods of scolopale cells. No expression is to be detected. (**A, B**) *control*^{18} immunostaining. (**C,D**) *atk*^{33} mutant.

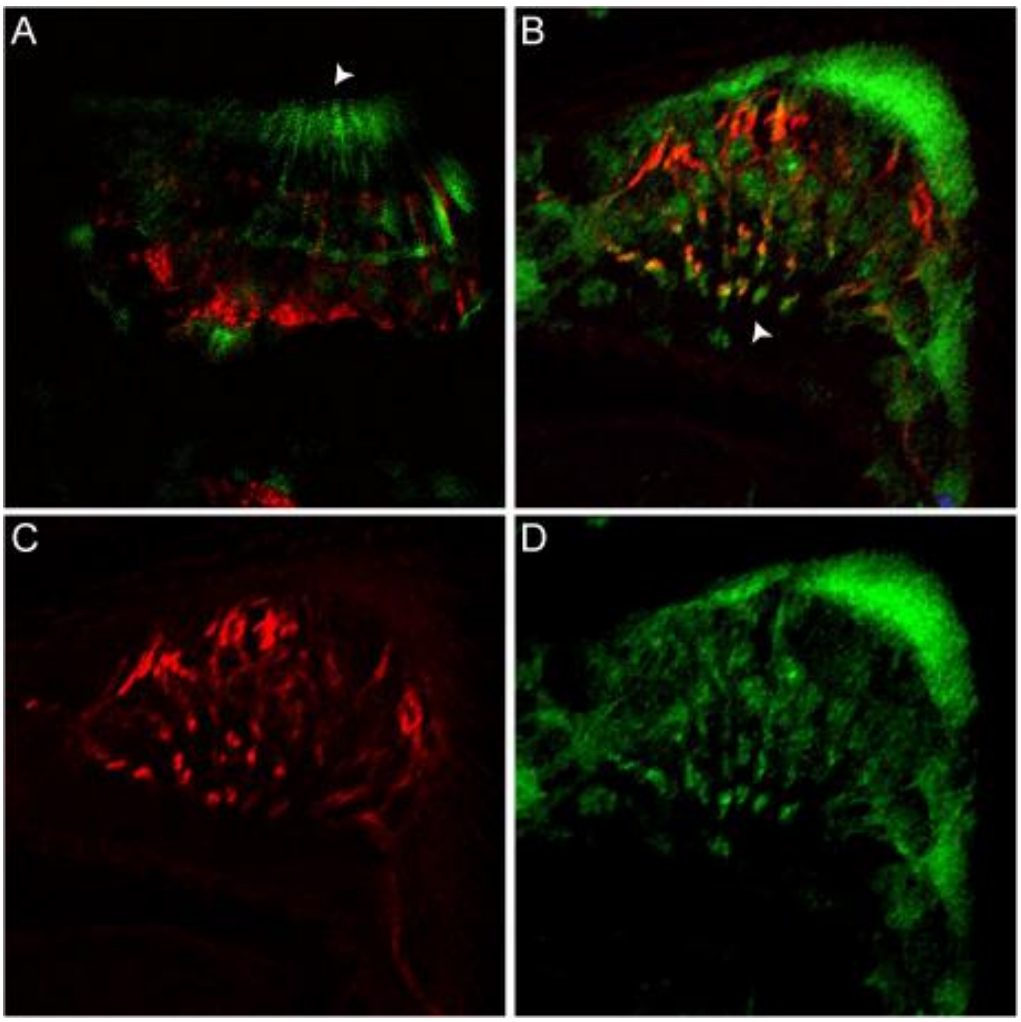

Figure 17: **Atk is present in the JO from late pupae.** WPP + 3d pupae JO immunostained with 22C10 (red) and anti-Atk (green). (**A, B**) Immunostaining of two different *white* pupae JO showing the localization of Atk at the tip of the sensory dendrites (arrowheads). The levels of the protein seems to be low. (**C,D**) Individual view of 22C10 staining (C) and anti-Atk staining (D).

4.8. Adult *atk* mutants do not show hearing impairments

The antennal sound receiver made up by the third antennal segment and the feather-like arista, acts as a simple spring-mass system that vibrates in response to the particle velocity component of sound (Gopfert, Robert 2001). When stimulated acoustically, the sound receiver moves and translates the vibration to the antennal joint to which the mechanosensory neurons are coupled. The mechanical force will alternately stretch and compress the mechanosensory neurons and thereby the mechanical stimulus will be transmitted (Göpfert, Robert 2003).

Vertebrate ears are able to amplify the mechanical input by pumping mechanical energy into the vibrations inside the ear. This mechanism is known as cochlear amplification and relies on the fact that some hair cells, the cochlear mechanosensory cells, apart from transducing the acoustic signal, are endowed with proteins that transform metabolic or electrical energy into mechanical energy that allow them to actively move and exert a positive feedback on the cochlear mechanism, thus improving the ear´s sensitivity to the small vibrations induced by faint sounds (Hudspeth, Gillespie, 1994).

As vertebrate hair cells do, *Drosophila* antennal ear also has shown to employ a positive feedback to positively amplify their sensitivity to sound. The mechanosensory neurons are motile and adapt their stiffness to compensate for the low intensities of sound (Göpfert, Robert 2003). By

measuring the response of the receiver to different sound-induced vibrations, it has been shown that its best frequency markedly moves when the stimulus intensity is altered (Göpfert, Robert 2003). Higher sound intensities (particle velocity ≥ ca. 1 mm/s) entail a best frequency (BF) of 800 Hz (Gopfert, Robert 2002; Göpfert, Robert 2003), while lower sound intensities shift the sound receiver BF down to ca. 150-200 Hz. This latter BF corresponds to the dominant frequency of the fly´s courtship song, suggesting that its sound receiver is tuned to conspecific songs (Nadrowski, Göpfert 2009). This intensity-dependant shift of the resonance frequency indicates stiffness nonlinearity and is dependent on the physiological condition of the animal, since dead and CO_2 anesthetized flies lose the non-linear response of their ear. Moreover, the nonlinearity of the fly ear associates with spontaneous oscillation activity that is also mechanosensory neuron dependent (Göpfert, Robert 2003). Finally, mechanosensory neurons in the JO exhibit power gain, as they add mechanical energy into the receiver´s fluctuations by expending biological energy (Goepfert et al. 2006) and thus amplifying the mechanical input of the ear.

Since *atk* is expressed during the pupal stages in the JO, and taking into account that lack of *atk* impairs the function of larval ch organs, we analyzed whether adult *atk* mutants also show hearing defects.

In a first attempt to analyze the hearing phenotype of *atk* mutants, we measured the free fluctuations of the arista in the absence of external

stimulation (Fig.18). These free fluctuations reflect both the passive motions due to thermal bombardment and active motions due to mechanical feedback from JO neurons (Gopfert et al. 2005). Usually in control flies, power spectra of the fluctuations show average best frequencies between 220 to 257 Hz, as our *control*^{18} flies do. Although *atk* mutants show a slightly lower best frequency (Fig.18B), but it is not significantly different from the control, indicating that *atk* mutants are not hearing impaired.

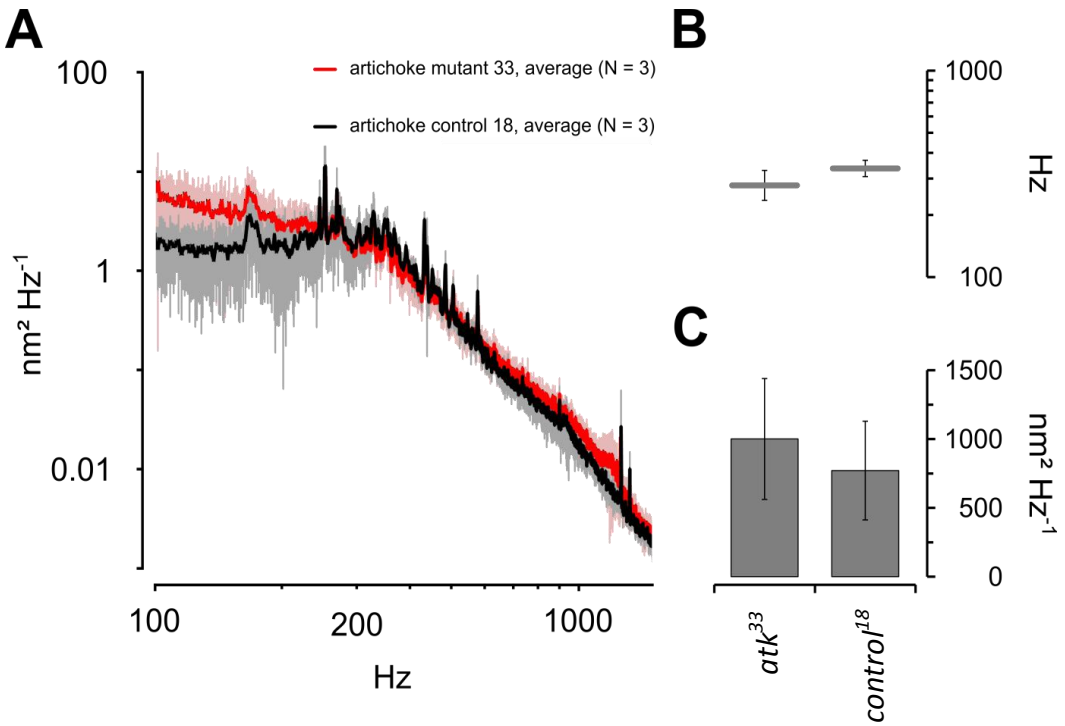

Figure 18: Mechanical responses of *atk* mutants. (**A**) Power spectra of the receiver´s vibrations measured in the absence of external stimulation. Red trace, average of the spectra obtained from 3 receivers of *atk* mutant flies. Light red traces, spectra obtained from *atk* mutant flies. Black trace, average of the spectra obtained from 3 receivers of *control*^{18} flies. Gray traces, spectra obtained from 3 receivers of *control*^{18} flies. There does not seem to be any difference between both dly strains. (**B**) Best frequency and (**C**) power spectral density of *atk* mutant and *control*^{18} flies. No significant difference appears between both phenotypes.

Discussion

DISCUSSION

Mechanotransduction is the less understood of the sensory modalities. Sensory signaling by light and chemicals is well described in molecular terms, but the molecules required for mechanosensation are barely known.

Since sensory terminals –consisting in many cases of cilium tips- are hardly exposed to the exterior world, mechanotransduction relies upon a proper connection between the cuticular structures that are receiving the mechanical energy, and the sensory neuron that will transduce it. Such connection is usually mediated by a supporting ECM. Although this ECM is thought to be essential for mechanotransduction by directly binding the mechanically-gated channels and transmitting them the mechanical forces, little is known about its molecular composition.

Much of the work concerning mechanosensory supporting ECM has been done in *C.elegans* (Chalfie, Au, 1989; Chalfie, Sulston, 1981; Emtage, 2004). So far, only the ZP-protein NompA has been described to localize to these ECM in *Drosophila*. However, the embryonic mechanosensory system of *D.melanogaster* represents an ideal model to study mechanosensation, since these organs are well characterized in terms of cellular composition, and localization and mutational screenings can be easily performed by simple behavioral assays (Hartenstein 1988; Kernan 2007; Caldwell et al.

2003). Moreover, comparative studies have shown that the genes orchestrating ciliogenesis are conserved among organisms (Avidor-reiss et al. 2004, Ben- Arie et al. 2000; Ernstrom and Chalfie, 2002; Gerdes et al. 2009).

In our study we have tried to shed some light on the organization of the supporting ECM by characterizing the novel LRR-protein Artichoke in *Drosophila melanogaster*. ATK immunostaining in *Drosophila* embryos shows that it is secreted into the ECM of ciliated sensory organs, where it localizes to the most distal region of the matrix. The presence of ATK in all ciliated sensory organs, its conservation among organisms with primary cilia (Avidor- Reiss et al. 2004) and the behavioral defects shown by *atk* mutants suggest a role for ATK in cilia assembly and function.

Our work additionally suggests that a proper coupling between the sensory cilium and the supporting ECM is also essential for chemotransduction, since *atk* mutants show chemosensitive defects. To our knowledge, these results provide the first experimental evidence for such an interaction.

5.1 *atk* is expressed in all ciliated sensory organs in the embryo, but the identity of the cell expressing it changes among them.

Our results show that *atk* is expressed in all type I sensory organs in the embryo, which encompass the chemo- and mechanosensory sensilla whose sensory perception relies on ciliated neurons. Despite their morphological differences, all ciliated sensory organs possess a supporting ECM and a common lineage program of 4 similar asymmetric divisions of the sensory organ precursors (SOP) that give rise to homologous cell types in each organ (Lai, Orgogozo 2004; Fichelson, Gho 2003; Orgogozo, Schweisguth & Bellaïche 2001) (Fig. 19).

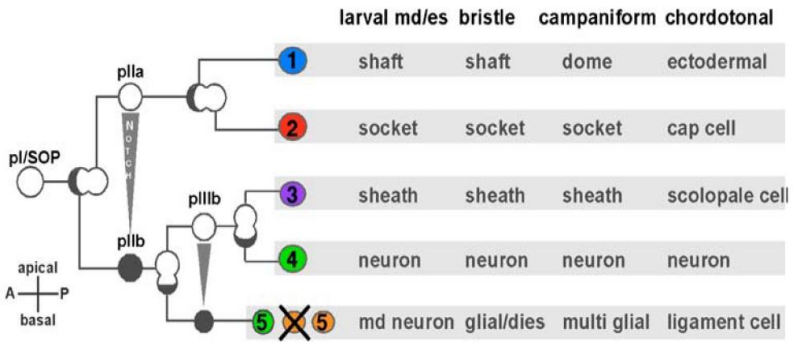

Figure 19: Sensory organ lineage and cell specification. A single SOP cell goes through four asymmetric mitotic divisions to give rise to the different cells that make up sensory organs, that are the neuron and the accessory cells, and, in some cases, a type II multidendritic (md) neuron. The diagram shows the orientation of the cell divisions as they occur along the apico-basal axis or in the plane of the epithelium. The shaded triangles indicate the orientation of the Notch signaling from a Notch signal-sender cell to a Notch signal-receiver cell. The fates of each of the cells resulting from the asymmetric divisions are listed for different organ types. This common lineage suggests that cells in different organ types are homologous one to one, even though morphologically different (Lai, Orgogozo, 2004).

A limited set of modifications of the core lineage, including changes in terminal cell fate, lineage proliferation, lineage apoptosis and cell recruitment, provides sensory organ diversity (Lai, Orgogozo 2004). The hypothesis of the existence of an ancestral ciliated sensory organ with the capacity of translating different stimuli that evolved into different sensory organ types is supported by this common lineage. Other evidences that reinforce this idea include recent studies that report the role played by chemoreceptor proteins and phototransducer components in *Drosophila* audition (Senthilan et al. 2012). All these sensory organs are specified by the basic helix-loop-helix (bHLH) proneural protein ATONAL (ATO), and they are thought to have evolved from a common "protosensory" organ that presumably looked like a ch organ (Niwa et al. 2004). The homologue of ATO in vertebrates, AtoH1, determines the specification of hair cells (Bermingham et al. 1999), so there seems to be a high degree of conservation among the sensory structures in very different organisms.

Since ATK is present in the supporting ECM of all ciliated sensory organs in *Drosophila*, it seems likely that it was already present in the presumptive ancestral sensory organ and has been preserved in the diversified sensory organs to play a similar function. Taking into account that primary cilia, such as the sensory cilia from *Drosophila*, appear in many organisms as sensory structures, we wondered whether ATK is conserved among them. Avidor-Reiss *et al*. (Avidor-Reiss et al. 2004) performed a comparative genomic analysis to find genes involved in ciliogenesis. Among them, they defined *atk* as a compartment gene, for being conserved among the

genomes of organsims such as *D. melanogaster*, *H.sapiens*, *C.elegans* and *T.brucei* that assemble at least some of their cilia through compartmentalized ciliogenesis, the process in which the cilium protrudes gradually from the basal bodies located under the plasma membrane. Most motile and primary cilia are generated following this ciliogenesis pattern. However, in a few cases the entire axoneme is first assembled in the cytosol, in a process termed cytosolic ciliogenesis. *atk* was not present in organism possessing only this type of ciliogenesis. Based on our results, we proposed, completing the conclusions from Avidor-Reiss, that ATK-like proteins should be involved in the process of attaching non-motile sensory cilia to support structures during the late development of sensory organs, but not directly in the process of cilia formation.

Going back to *atk* expression in *Drosophila*, even though ATK is present in es and ch organs, their cilia are anatomically and physiologically very different (Gong et al. 2004, Kim et al. 2003). Although ciliogenesis is controled in both cases by the *Drosophila* transcription factor Regulatory factor X (RFX) (Durand et al. 2000), differences arise as a consequence of the different gene regulation carried out by the proneural factors (Jarman, Ahmed 1998). There are two main subtypes of proneural proteins, the Atonal subclass (Ato and Amos) that specify the formation of ch organs (Jarman et al. 1993, Jarman et al. 1995), photoreceptors (Jarman et al. 1994) and some antennal chemosensory organs (Reddy et al. 1997), and the AS-C subclass (Achaete, Scute and Lethal of Scute) that promote the formation of es organs (Campuzano, Modolell 1992). In the paper from

Cachero *et al*. (Cachero et al. 2011) they observe that the ciliogenesis regulator *Rfx* is activated by different enhancers in es and ch organs. Our results show that the insertion of the P-element *Uhg8*^{EY07139} causes that *atk* is no longer expressed in ch organs, or at least not detectable by *in situ* hybridization procedures, and that the hypomorphic alleles *atk*^{8} and *atk*^{23} lose *atk* expression in mechanosensory organs from the body segments. This seems to indicate that there are also different enhancers controlling *atk* expression in different organ types, as it is the case for *Rfx*, and that these enhancers are affected by either the P-element *Uhg8*^{EY07139} insertion or by the small deletion in the hypomorphic alleles. These regulatory units could also explain the temporal and quantitative variations observed in *atk* expression between ch, es and chemosensory organs.

Another interesting issue is that *atk* is not expressed by homologous cell types in ch and es organs, but by "sister" cell types, namely by the cap cell in ch organs and the trichogen cell in es organs. These two types of cells are though described as homologous in terms of their function (Hartenstein 1988), since both are responsible for binding the sensory cilium to the stimulus-detecting structures. The specification program of these organs starts when groups of adjacent cells, termed proneural clusters, acquire neural potential within a field of undifferentiated epithelial cells due to the spatially patterned expression and activity of the proneural proteins (Lai, Orgogozo 2004). Within each proneural cluster, neural potential is restricted to a single cell, the single sensory organ precursor (SOP), which laterally inhibits the adjacent cells in a process

mediated by the Notch signaling pathway. Once stably determined, the SOP undergoes a series of 4 asymmetric cell divisions that are also regulated by the Notch pathway, giving rise to the different cells comprising an individual organ (Fig. 19). *atk* expression changes between the two daughter cells of the pIIa cell (Fig.19). In es organs, the transcription factor *D-Pax2* regulates the shaft cell differentiation program (Kaveler et al. 1999). By contrast, the transcription factors Supression of Hairless (SuH) and Sox15 repress *D-Pax2* expression in the socket cell to avoid the execution of the shaft differentiation program in this cell (Miller et al. 2009). However, these regulators are not expressed in ch organs, so that different regulators expressed either in ch or es organs that bind different enhancers could explain the shift in the *atk* expressing cell.

5.2 *atk* role in the assembly of ciliated mechanosensory organs

A supporting ECM has been described for most mechanosensory systems. Taking into account the very rapid transduction that occurs in mechanosensation, it is assumed that mechanotransduction channels should be directly mechanically-gated (Gillespie, Walker 2001). The prevailing model, based on studies on worm touch receptors (Chalfie 1997, Tavernarakis, Driscoll 1997) and vertebrate hair cells (Hudspeth, Gillespie 1994), states that the mechanotransduction channel is linked to both external and internal elastic gating springs that change the open

probability of the channel when external forces are transmitted to them through the ECM (Gillespie, Walker 2001).

The screening for mechanosensory mutants in *C.elegans* and *Drosophila* has always led to the identification of ECM molecules (Kernan 2007, Chalfie, Au 1989, Chalfie, Sulston 1981, Kernan, Cowan & Zuker 1994). Some of them, such as MEC-1 and MEC-5 proteins in *C.elegans*, directly bind the mechanotransduction channel and determine its localization (Emtage et al. 2004), while others, such as NompA in *Drosophila*, are thought to make up the ECM scaffold (Chung et al. 2001). Lack of any of these different types of proteins would lead to a disruption in the transmission of the mechanical stimuli and therefore mechanosensation would be impaired. Our results show that ATK localizes to the most distal region of the dendritic cap in mechanosensory organs, where it colocalizes with NompA along a thin band, but also extends its location above it. This indicates that ATK might be acting as a connector between the cilium tip and the apical structures, either the cap cell in ch organs or the bristle shaft in es organs. The morphological analysis on embryonic ch organs shows that cilia still connect to the dendritic cap (Fig.13I), but are not properly stretched and look loose compared to wild type cilia. Taking into account the transient expression of *atk* in mechanosensory organs during embryonic stages 15-16 and its posterior downregulation, and the anatomical defects of *atk* mutants, it seems more likely that ATK is involved in a developmental process common to all sensory cilia rather than directly in mechanotransduction, since in the latter case we would expect it to be

present during the larval stages when ch organs are functional. There are previous works that relate accessory cells to the development of their closely associated mechanosensory cilia. In the case of the v'ch1 organ, other embryonic ch organ located medio-laterally in each hemisegment, the cap cell strongly influences the orientation of the sensory cilium. Cap cell morphogenesis is at the same time regulated by the secreted protein Netrin-A, which is produced by epidermal cells to orientate the cap cell process that regulates sensory cilium positioning (Mrkusich et al. 2010).

The lch5 organ is the best characterized of all embryonic mechanosensory organs, so we made use of it to try to unravel ATK function. Lch5 precursors originate in the dorsal region of the embryo, but the mature organs are localized in the lateral PNS cluster. A two-step model has been proposed for this process: first, lch5 organs would rotate to assume a correct orientation; later, they would stretch ventrally to assume their final position, in a process mediated by the ligament cells (Inbal, Volk & Salzberg 2004, Inbal, Levanon & Salzberg 2003). After they acquire their final position, the dendritic cap is secreted during stages 15-16 by the scolopale (Hartenstein 1988) and cap cells (according to our findings), and the growing cilia should establish connections with this matrix. This phase coincides with the *atk* expressing temporal window. We propose that ATK is involved in this process, so that a lack of ATK would cause a defective binding of the cilium to the dendritic cap. Since ch organs are stretch receptors, all cells composing each scolopidium need to be tightened to transmit and sense the mechanical stimulus. An inadequate connection

between the cells would cause an anatomical defect because of the disruption of the tension between the cells. This is the phenotype that we observe in *atk* mutants: defective stretching in the sensory cilia and cap cells (Fig. 13H,I), and the resulting larval locomotive deficiencies. Considering that ATK is downregulated after stage 16 in mechanosensory organs, we suggest that it is required for the first targeting of the cilium to the ECM. Other proteins are probably responsible for the stabilization of this union before *atk* downregulates.

In the case of NompA, the only protein already described to localize to the dendritic cap, the mutant phenotype is even stronger since the sensory neurons seem to be separated from their apical connection site (Chung et al. 2001). In view of our co-immunostaining studies, it appears that NompA covers a broader space than ATK, which is only localized to the most distal region of NompA-localizing area. NompA possesses a ZP-domain typical of many components of specialized ECM (Chung et al. 2001, Mancuso et al. 2012, Plaza et al. 2010). It is expressed in both embryonic and larval stages and it is considered to make up the scaffolding of the dendritic cap, so it seems logical that whereas *NompA* mutants lose the contact between the cilium and the apical structures, in *atk* mutants this defect has smaller anatomical consequences.

Regarding to the precise location of ATK, both our experimental data and ATK amino acid sequence support that ATK is a secreted protein. It contains a signal sequence at the N-terminus and no recognizable

transmembrane domains (Smart Proteins). However, it contains a highly hydrophobic sequence of 10 amino acids at the C-terminus (ProScale) that might keep the protein inserted into the lipid bilayer, anchoring it to the cap cell membrane. The fact that ATK expands its localization beyond that of NompA supports this hypothesis. It is also possible that it binds to other membrane proteins by non-covalent interactions. Concerning ATK binding partner and taking into account that ATK is not expressed in the cilium membrane, we expect it to be a heterophilic interaction. Other LRR-proteins are also known to establish heterophilic interactions (Hong et al. 2009, Milan, Perez, AU, Cohen 2005). In the study from Cachero *et al*. (Cachero et al. 2011), where they try to identify genes implicated in sensory neuron differentiation that are regulated by the proneural transcriptional factor *atonal* (*ato*), they find two LRR-containing proteins, ATK and CG13125, which are regulated by ATO (Table S9). *CG13125* is expressed in the ch organs, as shown by the *in situ* hybridization experiments performed in this work, but it is not specified in which cells. It could be possible that CG13125 was the binding partner of ATK in the cilium membrane, but this hypothesis still needs to be investigated.

Previous work has identified ch organs as important components of the sensory feedback system required to elicit coordinated larval movements. Several mutations affecting ch organs show impaired locomotion (Caldwell et al. 2003). Our data show that all *atk* mutants present an uncoordinated larval locomotive behavior, indicating that these organs are not fully functional in *atk* mutants. Since *atk* is also expressed in es organs, we

cannot rule out the possibility that they are also influencing locomotion, but up to date it seems that ch organs and multidendritic neurons, where *atk* is not expressed, are the major contributors to the locomotive sensory feedback (Caldwell et al. 2003; Cheng et al. 2010). Moreover, *Uhg8*^{EY07139} homozygous larvae, which do not longer show *in situ* hybridization detectable expression in ch organs, but keep a detectable expression in es organs, do also show locomotive defects. Thus, the locomotion defects seen in *atk* mutants are most likely a consequence of the anatomical defects observed in ch organs. Our results therefore support the idea that a tight connection between the ECM and the neuronal membrane is necessary for the proper function of mechanosensory organs.

Concerning ATK role in adult sensory organs, the analysis of pupal JO indicates that ATK is also present in ch organs during pupal stages, but *atk* mutants do not show functional auditory impairments. Nevertheless, the requirement of an organized dendritic cap in adult ch organs is demonstrated by the evidence that *nompA* mutants show hearing impairments (Göpfert, Robert 2003). It seems thus possible that molecular redundancy camouflages Atk function.

5.3 *atk* role in chemosensory organs

Adult and larval chemosensory systems are anatomically very different. Although the organization of the adult chemosensory apparatus has been

described in detail, there are still a lot of questions concerning larval olfaction and gustation. The larval chemosensory system is composed of several organs. The dorsal organ (DO) seems to be a mixed organ for taste and smell and ends in a multiparous “dome”, whereas the terminal and the ventral organs (TO and VO respectively) respond to tastants only and are characterized by a terminal pore. The neuronal composition of the DO, TO and VO organs is well established in *Drosophila* (Stocker 2008), but less is known about the accessory cells or extracellular structures that support it. To our knowledge, an ECM has not yet been described for *Drosophila* chemosensory organs.

A detailed study on the larval chemosensory system of the house fly *Musca domestica* larval chemosensory system is available (Chu, Axtell 1971; Chuwang, Axtell 1972; Chuwang, Axtell 1972). In this work they describe in detail the fine anatomy of DO, TO and VO, including the accessory structures. In *Drosophila* we have only found one work describing the accessory cells of the DO, but only in terms of cell number and broad organization (Grillenzoni et al. 2007). However, since our results confirm the resemblance between *Musca* and *Drosophila* chemosensory systems, we have used the paper from Chu *et al*., to characterize ATK localization.

According to our results, *Drosophila* embryonic DO also contains a vacuolar-like structure surrounded by the olfactory cilia. ATK locates to this vacuole (Fig. 12D), which extends into the lumen of the olfactory dome. In *Musca*, the matrix within the vacuole is thought to be secreted by the

trichogen and tormogen cells. Since the trichogen synthesizes ATK, we also think that this is the case for *Drosophila.* The function of this vacuole is not known to our knowledge. Since it lies in the middle of the olfactory cilia, which travel a long distance from the cellular cell bodies to the olfactory dome, it probably plays a structural role, avoiding collapse of the cilia into each other. We also analyzed NompA localization. It is very interesting that NompA surrounds each cilium independently basally to Atk location, probably in the space between the scolopale cell and the cilium membrane (Fig 12D). These results indicate a separate function for ATK and NompA, which do not seem to interact in the DO. The fact that they colocalize in mechanosensory organs might be a consequence of these two proteins functioning in the same place, more than the result of a physical interaction between them. This issue needs to be further investigated.

In the case of the TO, ATK is located in the distal region of the NompA localizing area. Gustation is impaired in *atk* mutants, as shown by the behavioral assays, and this phenotype can be rescued when expressing a transgene carrying the genomic *atk* region in an *atk* mutant background. This implies that ATK is necessary for correct gustatory transduction.

Trying to decipher a role for ATK in chemosensation, we looked for anatomical defects in the chemosensory cilia of the DO, TO and VO. However, the lack of a stereotyped pattern of cilia organization in these organs made the detection of anatomical defects very difficult. Cilia travel from the cell bodies to the cuticular structures, either the dome or the

pore, in a hairy-like arrangement, so that subtle morphological defects might remain masked.

There are some works in the adult chemosensory system, especially olfaction, that attribute an important role for accessory cells in chemosensation. These cells secrete numerous odorant-binding proteins into the olfactory and taste lymph that are thought to bind ligands and present them to their receptors, but also to remove the ligands from the vicinity of the receptor site (Ebbs, Amrein 2007). However, as far as we know, ATK is the first protein described to be synthetized by the accessory cells performing a structural function. Since ATK also is present in mechanosensory cilia, we suggest that its role in chemosensation is not directly connected to the transduction of the stimulus, but to the assembly of the organ. The defects observed in *atk* mutants for the detection of sacarose are drastic. This suggests that the structure of the organ is severely disrupted in the mutants, probably because the accessibility to the pore is harshly reduced.

In summary, our results show that, as already known for mechanosensory organs, chemosensation is also dependent on the accessory structures that surround the sensory cilia, and that a supporting ECM is structurally required for a proper arrangement of these organs.

Conclusions

- *atk* is transiently expressed in all embryonic ciliated sensory organs in *Drosophila melanogaster.* However, the cell that synthetizes ATK varies: it is the cap cell in chordotonal organs, but the trichogen cell in external sensory and chemosensory organs. This implies a fine regulation of its expression.

- ATK is secreted to the supporting ECM located at the tip of the sensory cilia. In mechanosensory organs where this ECM is known as the dendritic cap, ATK localizes to the most distal region of the matrix. In chemosensory organs its localization changes depending on the organ type. Whereas it associates with the tip of the gustatory cilia, in olfactory organs it is secreted into the supporting vesicle that lies in the center of the organ.

- ATK deficiency causes defects in the mechanosensory cilia of the lch5 organ. These cilia are not properly stretched, probably due to an inadequate connection to the cuticular structures.

- Lack of ATK results in larval locomotion defects. This confirms previous results that ascribe a direct role of the dendritic cap in the mechanotransduction process, and suggests that the anatomical defects in *atk* mutants may result in a defective transmission of the mechanical stimuli to the mechanically-gated transduction channels.

- Lack of Atk also produces chemosensory defects. These results suggest that a supporting ECM is also indispensable for chemotransduction.

- *atk* is expressed in the JO during late pupal stages, when the adult peripheral nervous system is being remodeled.

- Lack of ATK does not seem to impair hearing. This could be due to the functional redundancy of ATK.

Conclusiones

- *atk* se expresa transitoriamente en todos los órganos sensoriales ciliados embrionarios en *D. melanogaster*. Sin embargo, el tipo de célula que lo sintetiza varía, siendo ésta la *cap cell* en los órganos cordotonales y la *trichogen cell* en los órganos sensoriales externos y en los órganos quimiosensoriales. Esto implica una regulación específica de su expresión en los distintos tipos de órganos.

- ATK es secretado a la matriz extracelular (MEC) de apoyo que se encuentra en la punta de los cilios sensoriales. En los cilios mecanosensoriales, donde esta MEC se conoce como *dendritic cap*, ATK se localiza en la región más distal de la matriz. En los órganos quimiosensoriales su localización cambia dependiendo del tipo de órgano. Mientras ATK se encuentra en la punta de cada uno de los cilios gustatorios, en los órganos olfatorios ATK es secretado a una vesícula que se encuentra en el medio del órgano y que queda rodeada por todos los cilios olfatorios.

- La falta de ATK causa defectos en los cilios mecanosensoriales del órgano lch5. Los cilios aparecen poco estirados probablemente debido a una conexión insuficiente con las estructuras cuticulares.

- La falta de ATK causa defectos en la locomoción larvaria. Ésto confirma resultados previos que atribuían a la *dendritic cap* un papel directo en el proceso de mecanotransducción y sugiere que los defectos anatómicos observados en los mutantes *atk* causan defectos en la transmisión del estímulo mecánico desde las estructuras cuticulares a los canales iónicos de apertura mecánica.

- La falta de ATK causa también defectos quimiosensoriales. Estos resultados implican que una MEC de soporte también es indispensable en el proceso de quimiotransducción.

- *atk* se expresa en el JO durante los estadios puparios tardíos, cuando el sistema periférico está siendo regenerado.

- La falta de ATK no causa defectos auditivos. Esto se puede deber a la redundancia funcional de ATK.

References

Affolter, M. & Basler, K. 2007, "The Decapentaplegic morphogen gradient: from pattern formation to growth regulation", *Nat Rev Genet,* vol. 8, no. 9.

Albert, J.T., Nadrowski, B. & Gopfert, M.C. 2007, "Mechanical signatures of transducer gating in the Drosophila ear", *Current Biology,* vol. 17, no. 11.

Albert, J.T., Nadrowksi, B. & Goepfert, M.C. 2007, "Drosophila mechanotransduction - Linking proteins and functions", *Fly,* vol. 1, no. 4.

Avidor-Reiss, T., Maer, A.M., Koundakjian, E., Polyanovsky, A., Keil, T., Subramaniam, S. & Zuker, C.S. 2004, "Decoding cilia function: defining specialized genes required for compartmentalized cilia biogenesis", *Cell,* vol. 117, no. 4.

Barolo, S., Walker, R.G., Polyanovsky, A.D., Freschi, G., Keil, T. & Posakony, J.W. 2000, "A notch-independent activity of suppressor of hairless is required for normal mechanoreceptor physiology", *Cell,* vol. 103, no. 6.

Bermingham, N.A., Hassan, B.A., Price, S.D., Vollrath, M.A., Ben-Arie, N., Eatock, R.A., Bellen, H.J., Lysakowski, A. & Zoghbi, H.Y. 1999, "Math1: An essential gene for the generation of inner ear hair cells", *Science,* vol. 284, no. 5421.

Blochlinger, K., Bodmer, R., Jan, L.Y. & Jan, Y.N. 1990, "Patterns of expression of cut, a protein required for external sensory organ development in wild-type and cut mutant Drosophila embryos", *Genes Dev,* vol. 4, no. 8.

Cachero, S., Simpson, T.I., Lage, P.I.Z., Ma, L., Newton, F.G., Holohan, E.E., Armstrong, J.D. & Jarman, A.P. 2011, "The gene regulatory cascade

linking proneural specification with differentiation in Drosophila sensory neurons", *PLoS Biol,* vol. 9, no. 1.

Caldwell, J.C., Miller, M.M., Wing, S., Soll, D.R. & Eberl, D.F. 2003, "Dynamic analysis of larval locomotion in Drosophila chordotonal organ mutants", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, no. 26.

Campuzano, S. & Modolell, J. 1992, "Patterning of the Drosophila Nervous-system - the Achaete-scute Gene-complex", *Trends In Genetics,* vol. 8, no. 6.

Chalfie, M. 1997, "A molecular model for mechanosensation in Caenorhabditis elegans", *Biological Bulletin,* vol. 192, no. 1, pp. Ctr Adv Studies S.

Chalfie, M. & Au, M. 1989, "Genetic-control of Differentiation of the Caenorhabditis-elegans Touch Receptor Neurons", *Science,* vol. 243, no. 4894.

Chalfie, M. & Sulston, J. 1981, "Developmental Genetics of the Mechanosensory Neurons of Caenorhabditis-elegans", *Developmental Biology,* vol. 82, no. 2.

Cheng, L.E., Song, W., Looger, L.L., Jan, L.Y. & Jan, Y.N. 2010, "The Role of the TRP Channel NompC in Drosophila Larval and Adult Locomotion", *Neuron,* vol. 67, no. 3.

Chu, I.W. & Axtell, R.C. 1971, "Fine Structure of Dorsal Organ of House Fly Larva, Musca-Domestica L", *Zeitschrift fur Zellforschung und Mikroskopische Anatomie,* vol. 117, no. 1.

Chung, Y.D., Zhu, J., Han, Y. & Kernan, M.J. 2001, "nompA encodes a PNS-specific, ZP domain protein required to connect mechanosensory dendrites to sensory structures", *Neuron,* vol. 29, no. 2.

Chuwang, I. & Axtell, R.C. 1972, "Fine-structure of Terminal Organ of House-fly Larva, Musca-domestica L", *Zeitschrift Fur Zellforschung Und Mikroskopische Anatomie,* vol. 127, no. 3.

Chuwang, I.W. & Axtell, R.C. 1972, "Fine-Structure of Ventral Organ of House-Fly Larva, Musca-Domestica L", *Zeitschrift fur Zellforschung und Mikroskopische Anatomie,* vol. 130, no. 4.

de Wit J., Hong,W., Luo, L. & Ghosh A. 2011 "Role of Leucine-Rich Repeat Proteins in the Development and Function of Neural Circuits", *Annu Rev Cell Dev Bio,* vol. 10, no. 27.

Dolan J., Walshe, K., Alsbury, S., Hokamp, K., O'Keeffe, S., Okafuji, T., Miller, S. F., Tear, G. & Mitchell, K.J. 2007 "The extracellular leucine-rich repeat superfamily; a comparative survey and analysis of evolutionary relationships and expression patterns", BMC Genomics, vol.14, no.8.

Dubruille, R., Laurencon, A., Vandaele, C., Shishido, E., Coulon-Bublex, M., Swoboda, P., Couble, P., Kernan, M. & Durand, B. 2002, "Drosophila regulatory factor X is necessary for ciliated sensory neuron differentiation", *Development,* vol. 129, no. 23.

Durand, B., Vandaele, C., Spencer, D., Pantalacci, S. & Couble, P. 2000, "Cloning and characterization of dRFX, the Drosophila member of the RFX family of transcription factors", *Gene,* vol. 246, no. 1-2.

Ebbs M.L. & Amrein, H. 2007 "Taste and pheromone perception in the fruit fly Drosophila melanogaster", *Pflugers Arch,* vol.454, no 5.

Eberl, D.F. & Boekhoff-Falk, G. 2007, "Development of Johnston's organ in Drosophila", *Int J Dev Biol,* vol. 51, no. 6-7.

Emtage, L., Gu, G.Q., Hartwieg, E. & Chalfie, M. 2004, "Extracellular proteins organize the mechanosensory channel complex in C-elegans touch receptor neurons", *Neuron,* vol. 44, no. 5.

Fichelson, P. & Gho, M. 2003, "The glial cell undergoes apoptosis in the microchaete lineage of Drosophila", *Development,* vol. 130, no. 1.

Gillespie, P.G. & Walker, R.G. 2001, "Molecular basis of mechanosensory transduction", *Nature,* vol. 413, no. 6852.

Goepfert, M.C., Albert, J.T., Nadrowski, B. & Kamikouchi, A. 2006, "Specification of auditory sensitivity Drosophila TRP channels", *Nature Neuroscience,* vol. 9, no. 8.

Gong, Z., Son, W., Chung, Y.D., Kim, J., Shin, D.W., McClung, C.A., Lee, Y., Lee, H.W., Chang, D., Kaang, B., Cho, H., Oh, U., Hirsh, J., Kernan, M.J. & Kim, C. 2004, "Two interdependent TRPV channel subunits, inactive and Nanchung, mediate hearing in Drosophila", *J Neurosci,* vol. 24, no. 41.

Gopfert, M.C. & Robert, D. 2002, "The mechanical basis of Drosophila audition", *Journal of Experimental Biology,* vol. 205, no. 9.

Gopfert, M.C. & Robert, D. 2001, "Turning the key on Drosophila audition", *Nature,* vol. 411, no. 6840.

Göpfert, M.C. & Robert, D. 2003, "Motion generation Drosophila mechanosensory neurons", *Proc Natl Acad Sci U S A,* vol. 100, no. 9.

Gopfert, M.C., Humphris, A.D.L., Albert, J.T., Robert, D. & Hendrich, O. 2005, "Power gain exhibited by motile mechanosensory neurons in Drosophila ears", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 102, no. 2.

Grillenzoni, N., de Vaux, V., Meuwly, J., Vuichard, S., Jarman, A., Holohan, E., Gendre, N. & Stocker, R.F. 2007, "Role of proneural genes in the formation of the larval olfactory organ of Drosophila", *Development Genes and Evolution,* vol. 217, no. 3.

Hartenstein, V. 1988, "Development of Drosophila larval sensory organs: spatiotemporal pattern of sensory neurones, peripheral axonal pathways and sensilla differentiation", *Development (Cambridge),* vol. 102, no. 4.

Hartenstein, V. & Campos-Ortega, J. 1985, "Fate-mapping in wild-typeDrosophila melanogaster", *Development Genes and Evolution,* vol. 194, no. 4.

Heimbeck, G., Bugnon, V., Gendre, N., Haberlin, C. & Stocker, R.F. 1999, "Smell and taste perception in Drosophila melanogaster larva: Toxin expression studies in chemosensory neurons", *Journal of Neuroscience,* vol. 19, no. 15.

Hong, W., Zhu, H., Potter, C.J., Barsh, G., Kurusu, M., Zinn, K. & Luo, L. 2009, "Leucine-rich repeat transmembrane proteins instruct discrete dendrite targeting in an olfactory map", *Nature neuroscience,* vol. 12, no. 12.

Hudspeth, A.J. & Gillespie, P.G. 1994, "Pulling Springs to Tune Transduction - Adaptation by Hair-Cells", *Neuron,* vol. 12, no. 1.

Inbal, A., Levanon, D. & Salzberg, A. 2003, "Multiple roles for u-turn/ventral veinless in the development of Drosophila PNS", *Development,* vol. 130, no. 11.

Inbal, A., Volk, T. & Salzberg, A. 2004, "Recruitment of ectodermal attachment cells via an EGFR-dependent mechanism during the organogenesis of Drosophila proprioceptors", *Dev Cell,* vol. 7, no. 2.

Jarman, A.P. & Ahmed, I. 1998, "The specificity of proneural genes in determining Drosophila sense organ identity", *Mech Dev,* vol. 76, no. 1-2.

Jarman, A.P., Grau, Y., Jan, L.Y. & Jan, Y.N. 1993, "atonal is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system", *Cell,* vol. 73, no. 7.

Jarman, A.P., Grell, E.H., Ackerman, L., Jan, L.Y. & Jan, Y.N. 1994, "Atonal is the proneural gene for Drosophila photoreceptors", *Nature,* vol. 369, no. 6479.

Jarman, A.P., Sun, Y., Jan, L.Y. & Jan, Y.N. 1995, "Role of the proneural gene, atonal, in formation of Drosophila chordotonal organs and photoreceptors", *Development,* vol. 121, no. 7.

Kalamajski, S. & Oldberg, A. 2010, "The role of small leucine-rich proteoglycans in collagen fibrillogenesis", *Matrix Biology,* vol. 29, no. 4.

Kamikouchi, A., Albert, J.T. & Göpfert, M.C. 2010, "Mechanical feedback amplification in Drosophila hearing is independent of synaptic transmission", *Eur J Neurosci,* vol. 31, no. 4.

Kavaler, J., Fu, W.M., Duan, H., Noll, M. & Posakony, J.W. 1999, "An essential role for the Drosophila Pax2 homolog in the differentiation of adult sensory organs", *Development,* vol. 126, no. 10.

Kernan, M.J. 2007, "Mechanotransduction and auditory transduction in Drosophila", *Pflugers Archiv-european Journal of Physiology,* vol. 454, no. 5.

Kernan, M., Cowan, D. & Zuker, C. 1994, "Genetic Dissection of Mechanosensory Transduction - Mechanoreception-defective Mutations of Drosophila", *Neuron,* vol. 12, no. 6.

Kim, J., Chung, Y.D., Park, D., Choi, S., Shin, D.W., Soh, H., Lee, H.W., Son, W., Yim, J., Park, C., Kernan, M.J. & Kim, C. 2003, "A TRPV family ion channel required for hearing in Drosophila", *Nature,* vol. 424, no. 6944.

Klein, Y., Halachmi, N., Egoz-Matia, N., Toder, M. & Salzberg, A. 2010, "The proprioceptive and contractile systems in Drosophila are both patterned EGR family transcription factor Stripe", *Dev Biol,* vol. 337, no. 2.

Kobe, B. & Kajava, A.V. 2001, "The leucine-rich repeat as a protein recognition motif", *Current opinion in structural biology,* vol. 11, no. 6.

Lai, E.C. & Orgogozo, V. 2004, "A hidden program in Drosophila peripheral neurogenesis revealed: fundamental principles underlying sensory organ diversity", *Dev Biol,* vol. 269, no. 1.

Laurençon, A., Dubruille, R., Efimenko, E., Grenier, G., Bissett, R., Cortier, E., Rolland, V., Swoboda, P. & Durand, B. 2007, "Identification of novel regulatory factor X (RFX) target genes comparative genomics in Drosophila species", *Genome Biol,* vol. 8, no. 9.

Lee, E., Sivan-Loukianova, E., Eberl, D.F. & Kernan, M.J. 2008, "An IFT-A protein is required to delimit functionally distinct zones in mechanosensory cilia", *Curr Biol,* vol. 18, no. 24.

Lee, J., Moon, S., Cha, Y. & Chung, Y.D. 2010, "Drosophila TRPN(=NOMPC) channel localizes to the distal end of mechanosensory cilia", *PLoS One,* vol. 5, no. 6.

Mancuso, V.P., Parry, J.M., Storer, L., Poggioli, C., Nguyen, K.C.Q., Hall, D.H. & Sundaram, M.V. 2012, "Extracellular leucine-rich repeat proteins are required to organize the apical extracellular matrix and maintain epithelial junction integrity in C elegans", *Development,* vol. 139, no. 5.

Matthews K.A., Miller, D.F. & Kaufman, T.C. 1990, "Functional implications of the unusual spatial distribution of a minor alpha-tubulin isotype in Drosophila: a common thread among chordotonal ligaments, developing muscle, and testis cyst cells". *Dev Biol.* vol.137, no.1.

Milan, M., Perez, L. & Cohen S.M. 2005 "Boundary formation in the Drosophila wing: functional dissection of Capricious and Tartan", *Dev Dyn*. vol 233, no.3.

Miller, S.W., Avidor-Reiss, T., Polyanovsky, A. & Posakony, J.W. 2009, "Complex interplay of three transcription factors in controlling the tormogen differentiation program of Drosophila mechanoreceptors", *Dev Biol,* vol. 329, no. 2.

Miyakawa, Y. 1982, "Behavioral Evidence for the Existence of Sugar, Salt and Amino-Acid Taste Receptor-Cells and some of their Properties in Drosophila Larvae", *Journal of insect physiology,* vol. 28, no. 5.

Mrkusich, E.M., Osman, Z.B., Bates, K.E., Marchingo, J.M., Duman-Scheel, M. & Whitington, P.M. 2010, "Netrin-guided accessory cell morphogenesis dictates the dendrite orientation and migration of a Drosophila sensory neuron", *Development,* vol. 137, no. 13.

Nadrowski, B. & Göpfert, M.C. 2009, "Level-dependent auditory tuning: Transducer-based active processes in hearing and best-frequency shifts", *Commun Integr Biol,* vol. 2, no. 1.

Orgogozo, V., Schweisguth, F. & Bellaïche, Y. 2001, "Lineage, cell polarity and inscuteable function in the peripheral nervous system of the Drosophila embryo", *Development,* vol. 128, no. 5.

Patel, N.H. 1994, "Imaging Neuronal Subsets and Other Cell-types In Whole-mount Drosophila Embryos and Larvae Using Antibody Probes", *Methods In Cell Biology,* vol 44.

Plaza, S., Chanut-Delalande, H., Fernandes, I., Wassarman, P.M. & Payre, F. 2010, "From A to Z: apical structures and zona pellucida-domain proteins", *Trends in cell biology,* vol. 20, no. 9.

Reddy, G.V., Gupta, B., Ray, K. & Rodrigues, V. 1997, "Development of the Drosophila olfactory sense organs utilizes cell-cell interactions as well as lineage", *Development,* vol.124, no. 3.

Senthilan, P.R., Piepenbrock, D., Ovezmyradov, G., Nadrowski, B., Bechstedt, S., Pauls, S., Winkler, M., Mobius, W., Howard, J. & Gopfert, M.C. 2012, "Drosophila auditory organ genes and genetic hearing defects.", *Cell,* vol. 150, no. 5.

Shishido, E., Takeichi, M. & Nose, A. 1998, "Drosophila synapse formation: regulation transmembrane protein with Leu-rich repeats, CAPRICIOUS", *Science,* vol. 280, no. 5372.

Singh, R.N. 1997, "Neurobiology of the gustatory systems of Drosophila and some terrestrial insects", *Microscopy research and technique,* vol. 39, no. 6.

Stocker, R.F. 2008, "Design of the larval chemosensory system", *Brain Development In Drosophila Melanograster,* vol. 628.

Swanson, L.E., Yu, M., Nelson, K.S., Laprise, P., Tepass, U. & Beitel, G. J. 2009, "Drosophila convoluted/dALS is an essential gene required for tracheal tube morphogenesis and apical matrix organization", *Genetics*, vol.181, no.4.

Tautz, D. & Pfeifle, C. 1989 "A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback". *Chromosoma*, vol. 98, no.2.

Tavernarakis, N. & Driscoll, M. 1997, "Molecular modeling of mechanotransduction in the nematode Caenorhabditis elegans", *Annual Review of Physiology,* vol. 59.

Vaessin, H., Grell, E., Wolff, E., Bier, E., Jan, L.Y. & Jan, Y.N. 1991, "Prospero is Expressed in Neuronal Precursors and Encodes a Nuclear-Protein that is Involved in the Control of Axonal Outgrowth in Drosophila", *Cell,* vol. 67, no. 5.

Vanvactor, D., Krantz, D.E., Reinke, R. & Zipursky, S.L. 1988, "Analysis of Mutants in Chaoptin, a Photoreceptor Cell Specific Glycoprotein in Drosophila, Reveals its Role in Cellular Morphogenesis", *Cell,* vol. 52, no. 2.

Wayburn, B. & Volk, T. 2004, "LRT, a tendon-specific leucine-rich repeat protein, promotes muscle-tendon targeting through its interaction with Robo". *Development*, vol. 136, no.21.

Yack, J.E. 2004, "The structure and function of auditory chordotonal organs in insects", *Microsc Res Tech,* vol. 63, no. 6.